

Best Available Copy

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



B 1

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A01N 63/00, A61K 48/00, 35/34, C12N 5/00		A1	(11) International Publication Number: WO 96/18303	
			(43) International Publication Date: 20 June 1996 (20.06.96)	
(21) International Application Number: PCT/US95/16093		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN. European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).		
(22) International Filing Date: 13 December 1995 (13.12.95)		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN. European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).		
(30) Priority Data: 08/354,944 13 December 1994 (13.12.94) US 08/477,377 7 June 1995 (07.06.95) US		(71)(72) Applicant and Inventor: LAW, Peter, K. [CA/US]; Suite 18, 1770 Moriah Woods Boulevard, Memphis, TN 38117 (US). (74) Agent: KENNEDY, Pat, Winston; Rhodes Coats & Bennett, L.L.P., P.O. Box 2974, Greensboro, NC 27402 (US).		
(54) Title: MYOBLAST THERAPY FOR MAMMALIAN DISEASES		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>		
(57) Abstract <p>Compositions and methods of treating mammalian diseases using myoblasts, and/or their physical, genetic, chemical derivatives. Myogenic cells that are normal, or genetically or phenotypically altered are cultured and transplanted into malfunctioning and/or degenerative tissues or organs to alleviate conditions that are hereditary, degenerative, debilitating, undesirable, and/or fatal. Treatment of these conditions is not limited to the usage of mechanical, electrical or physical properties of these myogenic cells, but includes the usage of biochemicals secreted/released by the latter. The present invention discloses the use of normal myoblasts to deliver the complete normal genome to effect genetic repair, or to augment the size, or the function of tissues or organs. Certain conditions may be better served with genetically altered myogenic cells derived from gene transduction, whereas others may be better served with cytoclimes converter cells. Endogenous biochemical(s) are used to control cell fusion of myoblasts among themselves or with other cell types. An automated cell processor within a cell bank which enables the manufacture, at a single run, of unprecedented large quantities (greater than 100 billion) of normal or genetically or phenotypically altered myogenic cells is also disclosed.</p>				

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	L1	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

MYOBLAST THERAPY FOR MAMMALIAN DISEASES**REFERENCE TO RELATED APPLICATION**

5 This application is a continuation-in-part of U.S. Application Serial No. 08/354,944, filed December 13, 1994. This reference is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION**10 FIELD OF THE INVENTION**

The present invention relates to compositions and methods for treating mammalian disease conditions that are debilitating, fatal, hereditary, degenerative and/or undesirable. More specifically, the present invention relates to the transplantation of normal, or genetically transduced, or cytocline-converted myogenic cells into malfunctioning, and/or degenerative tissues or organs.

20 DESCRIPTION OF THE PRIOR ART**MYOBLAST PROPERTIES**

In mammals, myoblasts are the only cell type which divide extensively, migrate, fuse naturally to form syncytia, lose their major histocompatibility Class I (MHC 1) antigens soon after fusion, and develop to occupy 25 50% of the body weight in humans. These combined properties render myoblasts ideal for gene transfer and somatic cell therapy (SCT). Myoblast therapy is a combined SCT and gene therapy.

MYOBLAST THERAPY

30 Although the role of myoblasts/satellite cells in myogenesis and muscle regeneration dated back to the early 1960s (Konigsberg, I.R., Science, 140:1273 (1963). Mauro, A. J., Biophys. Biochem. Cytol., 9:493-495 (1961)), their use in animal therapy was not reported 35 until 1978 (Law, P.K., Exp. Neurol., 60:231-243 1978)).

The first myoblast transfer therapy (MTT) on a Duchenne muscular dystrophy (DMD) boy on February 15, 1990 marked the first clinical trial on human gene transfer. Its success was reported (Law, P.K. et al.,

Lancet, 336:114-115 (1990); Kolata, G. The New York Times, Sunday, (June 3, 1990)). Unlike bone marrow transplant which strictly replaces genetically abnormal cells with normal ones, MTT actually inserts, through natural cell fusion, all the normal genes within the nuclei of the donor myoblasts into the dystrophic myofibers to repair them. In addition, donor myoblasts also fuse among themselves, forming genetically normal myofibers to replenish degenerated ones. Thus, full complements of normal genes are integrated, through a natural developmental process of regeneration, into the abnormal cells and into the abnormal organ.

The US Patent Office issued to this inventor a patent (#5,130,141) entitled "Composition for and methods of treating muscle degeneration and weakness" on July 14, 1992.

In October, 1993, the Food and Drug Administration (FDA) officially began regulating somatic cell therapy (SCT) with a definition of "autologous, allogenic, or 20 xenogeneic cells that have been propagated, expanded, selected, pharmacologically treated, or otherwise altered in biological characteristics ex vivo to be administered to humans and applicable to the prevention, treatment, cure, diagnosis, or mitigation of disease or injuries." 25 (Federal Register, 58:53248-53251 (1993)).

MTT falls under the umbrella of SCT and myoblasts and its physical, genetic or chemical derivatives become potential biologics in the treatment of mammalian diseases.

As of May 25, 1994 the FDA has granted permission for 30 Cell Therapy Research Foundation (CTRF) to charge \$63,806 per subject. CTRF is an non-profit 501 (c) (3) research foundation founded by the inventor in 1991. Authorization by the FDA for CTRF to recover costs from subjects of these 35 clinical trials is extremely important to establish the scientific credibility MTT and CTRF deserve, quoting the June 17, 1994 edition of the Memphis Health Care News,

"Permission to bill for an Investigational product is granted rarely," says FDA spokesman Monica Revelle, "Applicants must endure numerous procedures, and must have what looks like a viable product at the end of the rainbow. 5 It's used mainly to support testing of promising technology by small companies." This statement was made in regard to research at CTRF.

At this time CTRF holds the first and only 10 FDA-approved human clinical trial under an Investigational New Drug (IND) application on MTT. It is extremely important to realize that CTRF has been working closely with the FDA to establish criteria and policies in the approval process of this IND for genetic cell therapy. The 15 use of viral vector mediated gene therapy on human neuromuscular diseases has not met FDA approval.

CELL THERAPY WITH MYOBLASTS

The cell is the basic unit of all lives. It is that 20 infinitely small entity which life is made of. With the immense wisdom and knowledge of the human race, we have not been able to produce a living cell from nonliving ingredients such as DNA, ions, and biochemicals.

Cell Culture is the only method known to man for the 25 replication of cells *in vitro*. With proper techniques and precautions, normal or transformed cells can be cultured in sufficient quantity to repair, and to replenish degenerates and wounds.

Cell transplantation bridges the gap between *in vitro* and *in vivo* systems, and allows propagation of "new life" 30 in degenerative tissues or organs of the living yet genetically defective or injured body.

Cell fusion transfers all the normal genes within the nucleus like delivering a repair kit to the abnormal cell. It is important to recognize that, for proper installation 35 and future operation, the software packaged in the chromosomes needs other cell organelles as hardware to operate.

Correction of a gene defect occurs spontaneously at the cellular level after cell fusion. The natural integration, regulation and expression of the full complement of over 80,000 normal genes impart the normal phenotypes onto the heterokaryon. Protein(s) or factor(s) that were not produced by the host genome because of the genetic defect are now produced by the donor genome that is normal. Various cofactors derived from expression of the other genes corroborate to restore the normal phenotype.

10

GENE THERAPY WITH MYOBLASTS

The use of myoblasts as gene transfer vehicles has been researched by this inventor extensively. In mammals, myoblasts are the only cell type which divide extensively, migrate, fuse naturally to form syncytia, lose MHC-1 antigens soon after fusion, and develop to occupy 50% of the body weight in humans. These combined properties render myoblasts ideal for gene transfer.

Natural transduction of normal nuclei ensures orderly replacement of dystrophin and related proteins at the cellular level in DMD. This ideal gene transfer procedure is unique to muscle. After all, only myoblasts can fuse and only muscle fibers are multinucleated in the human body. By harnessing these intrinsic properties, MTT transfers all normal genes to effect genetic repair. Since donor myoblasts also fuse among themselves to form normal fibers in MTT, the muscles benefit from the addition of genetically normal cells as well.

30

MYOBLAST THERAPY IS THE MEDICINE OF THE FUTURE

Health is the well-being of all body cells. In hereditary or degenerative diseases, sick cells need repairing and dead cells need replacing for health maintenance.

Cell culture is the only way to generate new, live cells that are capable of surviving, developing and

functioning in the body after transplantation, replacing degenerated cells that are lost.

Myoblasts are the only cells in the body capable of natural cell fusion. The latter allows the transfer of all of the normal genes into genetically defective cells to effect phenotypic repair through complementation. MTT on DMD is the first human gene therapy demonstrated to be safe and effective. The use of MTT to transfer any other genes and their promoters/enhancers to treat other forms of diseases is underway. Myoblasts are efficient, safe and universal gene transfer vehicles, being endogenous to the body. Since a foreign gene always exerts its effect on a cell, cell therapy will always be the common pathway to health. After all, cells are what life is made of.

15

DMD: A SAMPLE DISEASE

DMD is a hereditary, degenerative, debilitating, fatal, and undesirable mammalian disease. It is characterized by progressive muscle degeneration and loss of strength. These symptoms begin at 3 years of age or younger and continue throughout the course of the disease. Debilitating and fatal, DMD affects 1 in 3300 live male births, and is the second most common lethal hereditary disease in humans. DMD individuals are typically wheelchair-bound by age 12, and 75% die before age 20. Pneumonia usually is the immediate cause of death, with underlying respiratory muscle degeneration and failure of DMD individuals to inhale sufficient oxygen and to expel lung infections. Cardiomyopathic symptoms develop by mid-adolescence in about 10% of the DMD population. By age 18, all DMD individuals develop cardiomyopathy, but cardiac failure is seldom the primary cause of death.

Before 1950, over 80 chemicals were evaluated and 33 were reported as potentially beneficial (Milhorat, A.T., *Medical Annals of the District of Columbia*, 23:15 (1954)). None are currently being used (Wood, D.S., In:

Kakulas, B.A. and Mastaglia, F.L., eds.: *Pathogenesis and therapy of Duchenne and Becker muscular dystrophy*. New York: Raven Press; 85-99 (1990)). Unconfirmed therapeutic benefits in DMD have been reported with 5 vitamins, amino acids (Van Meter, J.R., *Calif. Med.*, 79:297 (1953)), ATP (Nakahara, M., *Arzneim. Forsch.*, 15:591(1965)), coenzyme Q (Folkers, K. et al., *Excerpta Med. Int. Congr. Ser.*, 334:158 (1974)), adenylosuccinic acid (Bonsett, C.A., *Indiana Medicine*, 79:236 (1986)) and 10 growth hormone inhibitor (Coakley, J.H. et al., *Lancet*, 1(8578): 184 (1988)). Several hundred drugs have been screened (Wood, *supra*), with some studies showing consistent benefits from steroids (Entriakin, R.K. et al., *Muscle Nerve*, 7:130-136 (1984)).

15 The beneficial effects of prednisone on DMD was first reported almost 20 years ago (Drachman, D.B. et al., *Lancet*, 2:1409-1412 (1974)). The researchers reported that prednisone could delay the degenerative process and in some cases even transiently strengthen DMD 20 muscles. The evidence substantiating prednisone is not without debate (see Munsat, T.L. and Walton, J.N., *Lancet*, 1:276-277 (1985); Rowland, L.P., *Lancet*, 1:277 (1975); and Siegel, I.M. et al., *I.M.J.*, 145:32-33 (1974)). Although the mechanism(s) through which 25 prednisone mediates its effect is undefined. Prednisone causes numerous side-effects, and prolonged use induces adverse reactions that by far out-weigh the questionable benefits reported.

Gene manipulation and transfer are other approaches 30 that are being used to treat hereditary and degenerative diseases. However, it will be quite some time before this type of treatment finds clinical application for DMD (Law, P.K., In: Kakulas, B.A. et al., eds.: *Pathogenesis and therapy of Duchenne and Becker muscular dystrophy*. New York: Raven Press, 190 (1990); and Watson, J.D. et 35 al., *Recombinant DNA*. New York: W.H. Freeman and Co.; 576 (1992)). Success claimed over intramuscular DNA

injections (Acsadi, G. et al., *Nature*, 352: 815-818 (1991); and Wolff, J.A. et al., *Science*, 247:1465-1468 (1990)) and arterial delivery of immature muscle cells, also known as myoblasts, to skeletal muscle (Neumeyer, A.M. et al., *Neurology*, 42:2258-2262 (1992)) is very limited and questionable. Attempt of using transfected autologous myoblasts has resulted in low efficiency and mutation in transfection (Barr, E. and Leiden, J.M., *Science*, 254: 1507-1509 (1991); Dhawan, J. et al., *Science*, 254: 1509-1512 (1991); and Smith, B.F. et al., *Mol. Cell. Biol.*, 10: 3268-3271 (1990)). Such approach will yield insufficient myogenic cells to provide for a whole body myoblast transfer therapy (MTT) to treat DMD patients (Law, *supra*). Clinical trials are currently underway for cystic fibrosis (CF) based on transgenic mice studies (Hyde, S.C. et al., *Nature*, 362: 250-255- (1993)). Clinical trials with gene therapy have also been attempted on severe combined immunodeficiency (SCID). Unlike CF and SCID whose genetic defects are mediated through enzymic deficiencies, the genetic defect of DMD manifested as the absence of a structural protein called dystrophin in the cell membrane rather than a regulatory protein.

Although dystrophin serves as a good genetic/biochemical marker (Hoffman, E.P. et al., *Cell*, 51: 919-928 (1987)) in the evaluation of muscle improvements, dystrophin replacement constitutes only part of the treatment process. This has already been demonstrated, among others, by the present inventor using MTT in mdx mice (Chen, M. et al., *Cell Transplantation*, 1:17-22 (1992); Karpati, G. et al., *Am. J. Pathol.*, 135: 27-32 (1989); and Patridge, T.A., et al., *Nature*, 337:176-179 (1989)) and in humans (Gussoni, E., et al., *Nature*, 356: 435-438 (1992); Huard, J. et al., *Clin. Sci.*, 81:287-288(1991); Huard, J. et al., *Muscle Nerve*, 15:550-560 (1992); Law, P.K. et al., *Lancet*, 336:114-115 (1990); Law, P.K. et al., *Acta Paediatr. Jpn.*, 33:206-215

(1991); Law, P.K. et al., *Adv. Clin. Neurosci.*, 2:463-470 (1992); Law, P.K. et al., In: Angelini, C. et al., eds. *Muscular dystrophy research*. New York: Elsevier Science Publishers, 109-116 (1991); and Law, P.K. et al., *Acta Cardiomiologica*, 3:281-301 (1991)). Because DMD pathology is one of muscle degeneration and weakness, structural and especially functional improvements are of primary concern. These two parameters have been extensively studied using the dy^{2J}dy^{2J} dystrophic mouse as an animal model of hereditary muscle degeneration (Law, P.K., *Exp. Neurol.*, 60:231-243 (1978); Law, P.K., *Muscle Nerve*, 5:619-627 (1982); Law, P.K. et al., *Transplant Proc.*, 20:1114-1119 (1988); Law, P.K. et al., In: Griggs, R.C.; Karpati, G., eds. *Myoblast Transfer Therapy*. New: Plenum Press; 75-87 (1990); Law, P.K. et al., *Muscle Nerve*, 11:525-533 (1988); Law, P.K. et al., In: Kakulas, B.A.; Mastaglia, F.L., eds. *Pathogenesis and therapy of Duchenne and Becker muscular dystrophy*. New York: Raven Press; 101-118 (1990); and Law, P.K. and Yap, J.L., *Muscle Nerve*, 2:356-363 (1979)). These studies lead to the first MTT clinical trial or single muscle treatment (SMT) (Gussoni, E. et al., *Nature*, 356:435-438 (1992); Huard, J. et al., *Clin. Sci.*, 81:287-288 (1991); Huard, J. et al., *Muscle Nerve*, 15:550-560 (1992); Law, P.K. et al., *Lancet*, 336:114-115 (1990); Law, P.K. et al., *Acta Paediatr. Jpn.*, 33:206-215 (1991); Law, P.K. et al., *Adv. Clin. Neurosci.*, 2:463-470 (1992); Law, P.K. et al., In: Angelini, C. et al., eds. *Muscular dystrophy research*. New York: Elsevier Science Publishers: 109-116 (1991); and Law, P.K. et al., *Acta Cardiomiologica* 3:281-301 (1991)).

The feasibility, safety, and efficacy of MTT were assessed by this inventor in experimental lower body treatments involving 32 DMD boys aged 6-14 years of age, half of whom were non-ambulatory (Law, P.K. et al., *Cell Transplantation*, 2; 485-505 (1993)). Through 48 injections, 5 billion ($55.6 \times 10^6/\text{mL}$) normal myoblasts

were transferred into 22 major muscles in both lower limbs in each of the subjects. Results at 9 months after MTT indicated, interalia, that (1) MTT is a safe treatment; (2) MTT improves muscle function in DMD; and (3) more than 5 billion myoblasts are necessary to strengthen both lower limbs of a DMD boy between 6 and 14 years of age.

OTHER DISEASE CONDITIONS

Potentially every genetic disease can be benefited by MTT. Through natural cell fusion, donor myoblasts insert full complement of normal genes into genetically abnormal cells to effect repair. Promoters and enhancers of the defective gene can be supplied or activated or repressed to achieve gene transcription and translation with the release of hormone(s) or enzyme(s) from transplanted myogenic cells. Likewise, structural protein(s) can be produced to prevent or to alleviate disease conditions.

Alternatively transduced myoblasts can be used. The procedure consists of a) obtaining a muscle biopsy from the patient, b) transfecting a "seed" amount of satellite cells with the normal gene, c) confirming the myogenicity of the transfected cells, d) proliferating the transfected myoblasts to an amount enough to produce beneficial effect and e) administering the myoblasts into the patient.

Retroviral vectors have been used to transfer genes into rat and dog myoblasts in primary cultures under conditions that permit the transfected myoblasts to differentiate into myotubes expressing the transferred genes (Smith, B.F. et al., Mol. Cell Biol., 10:3268-3271 (1990)). Furthermore, mice injected with murine myoblasts that are transfected with human growth hormone (hGH) show significant levels of hGH in both muscle and serum that are stable for 3 months (Dhawan, J. et al.,

Science, 254:1509-1512 (1991); Barr E. and J.M. Leiden, Science, 254:1507-1509 (1991)).

The transduced myoblast transfer was inspired by a similar approach on adenosine deaminase (ADA) deficiency.

5 In the latter situation, T cells from the patient were transfected with functional ADA genes and returned to the patient after expansion in the number of the transfected cells through cell culture (Culver, K.W. et al., Transpl. Proc., 23:170-171 (1991)).

10 Similar approach has already been tested in animals using genetically transduced myoblasts to treat hemophilia B (Yao, S.N. et al., Gene Therapy, 1:99-107 (1994)), cardiomyopathy (Marelli, D., Cell Transplantation, 1:383-390 (1992); Koh, G.Y. et al., J. Clin. Inves., 92:1548-1554 (1993)), anemia (Hamamori, Y. et al., Human Gene Therapy, 5:1349-1356 (1994)). Undoubtedly, there will be many hereditary diseases to which myoblast therapy will apply.

15 20 25 30 Although differentiated, myoblasts are nonetheless embryonic cells that are capable of de-differentiated or even converted. Thus, myoblasts have recently been shown to be converted into osteoblasts with bone morphogenetic protein-2 (Katagiri, T. et al., J. Cell Biol., 127:1755-1766 (1994)). This study demonstrates that cytocline-converted myoblasts can be administered to patients with bone/cartilage degenerative diseases. Alternatively, it has been demonstrated that mouse dermal fibroblasts can be converted to a myogenic lineage (Gibson, A.J. et al, J. Cell Sci., 108:207-214 (1995)). The implicated usage of myoblast transfer therapy to treat cancer and type II diabetes mellitus is described below.

WHY MYOBLAST THERAPY

35 In hereditary or degenerative diseases, gene defects cause cells to degenerate and die with time. An effective treatment must not only repair degenerating

cells, but replenish dead cells as well. This can best be achieved by the transplantation of genetically normal cells, or somatic cell therapy. The advent of molecular genetics favors single gene manipulation which is currently being explored to treat genetic diseases. Like pharmaceuticals, single gene manipulation cannot replenish lost cells. Further, there is very limited evidence that these approaches can repair degenerating cells.

In U.S. Patent No. 5,130,141, this inventor disclosed for the first time compositions and methods for treating muscle degeneration and weakness. A composition comprised of genetically normal myoblasts from a donor was injected into one or more of the muscles of a host having a hereditary neuromuscular disorder. Muscle structure and function were greatly improved with the injection, thereby preventing or reducing muscle weakness which is a primary cause of crippling and respiratory failure in hereditary muscular dystrophies. This transplantation of genetically normal muscle cells into the diseased muscles of patients with hereditary muscular dystrophy is known as MTT.

MTT differs significantly from the conventional single gene transfer format in several respects. In this latter gene therapy, single copies of the down-sized dystrophin gene are transduced as viral conjugates into the mature dystrophic myofibers in which many proteins, both structural and regulatory, are lost previously. Multiple gene insertion is necessary to replace these lost proteins (Fig. 1). More gene insertion is needed to produce the cofactors to regulate and to express these lost proteins in order to repair the degenerating cell.

SUMMARY OF THE INVENTION

The demonstration of preliminary feasibility, safety, and efficacy (Law et. al., *Cell Transplantation*, 2:485 (1993)) of myoblast transfer therapy MTT prompted

this inventor to initiate a whole body trial (WBT) injecting 25 billion myoblasts into each of 64 Duchenne muscular dystrophy (DMD) boys and a boy with infantile facioscapulohumeral dystrophy (IFSH). The randomized 5 double-blind clinical trial protocol, approved by the FDA (IND Phase II) and the Essex IRB involves two MTT procedures separated by 3 to 9 months. Each procedure delivers 200 injections or 12.5 billion myoblasts, to either 28 muscles in the upper body (UBT) or to 36 10 muscles in the lower body (LBT). Injected muscles include those in the neck, shoulder, back, chest, abdomen, arms, hips, and legs. Subjects take oral cyclosporine for 3 months after each MTT. One IFSH and 10 DMD boys have received WBT and 20 more DMD boys have 15 received UBT or LBT in the past 17 months with no adverse reaction. These preliminary results indicate that the WBT is feasible and safe. While blinding will continue until the end of the study as to which side of the biceps brachii or quadriceps received myoblasts or placebo, five 20 subjects have demonstrated immunocytochemical evidence of dystrophin in one of these muscle biopsies 3 to 9 months after MTT. The contralateral muscle biopsies show no dystrophin. The pulmonary function (FVC) either shows no deterioration, or has improved by 15 to 25% in over 80% 25 of the subjects 3 to 6 month after MTT. About 50% of the subjects report behavioral improvement in running, balancing, climbing stairs and playing ball. One 14 yr-old DMD subject has stayed active without the need of a wheelchair after MTT (Law, P.K. et al., Amer Soc Neural 30 Transpl Abst., p. 27 (1995); Law, P.K. et al., J. Cellular Biochem. Supp, 21A:367, (1995)).

This demonstration of feasibility and safety in administering 30 billion myoblasts into a human subject provides the pivotal evidence that myoblasts can be used 35 as a biologic to treat human diseases. The demonstration of the dosage effectiveness further confirms the idea that myoblast therapy can be used to treat a whole

variety of mammalian diseases be it a hereditary, degenerative, debilitating, fatal, or undesirable disease condition.

The present invention provides compositions and methods for repairing degenerating cells and replenishing lost cells in patients with hereditary or degenerative diseases, in particular those characterized by muscle malfunction, degeneration and weakness. In practicing the present invention, any myogenic cell may be used, regardless of whether it is of skeletal, smooth, or cardiac in origin. Transferred cell types include myoblasts, myotubes and/or young muscle fibers. The myogenic cells may be primary-cultured or cloned from muscle biopsies of normal donors. They may also be cytocline converted or genetically transduced myogenic cells. Typically, the parents, siblings, or friends of the dystrophic patient are the donors. In addition, it is contemplated that the establishment of superior cell lines of myoblasts, whether from humans or animals, will provide a ready access of healthy donor cells for patients who do not otherwise have a suitable donor (Fig. 2 to 5, also Law, P.K., *Myoblast Transfer*, Landes, Austin, (1994)). It is further contemplated that the cell transplantation procedure will augment size, shape, appearance or function, and/or alleviate the disease conditions.

The present invention provides a method for controlling, initiating, or facilitating cell fusion once the myoblasts are injected into one or more of the muscles of a patient with the degenerative disease. By artificially increasing the concentration of the large chondroitin-6-sulfate proteoglycan (LC6SP) over the patient's endogenous level, fusion of the transferred donor myoblasts among themselves or with other cell types can be enhanced and controlled. (*Law, P.K., Myoblast Transfer Landes, Austin (1994)*).

It is yet another object of the invention to improve the fusion rate between the host and donor cells. To this end, various injection methods were tested and compared including injecting diagonally through the myofibers, perpendicular to the myofiber surface, parallel to the myofibers, and at a single site into the muscle. The goal is to achieve maximum cell fusion with the least number of injections (Figs. 6 to 8, also Law, P.K., *Myoblast Transfer*, Landes Austin, (1994)).

In a further embodiment, the technologies of *in vitro* fertilization and blastomere recombination can be used on known Duchenne carriers to increase their chances of having normal children (Fig. 9 to 13; also Law, P.K., *Myoblast Transfer*, Landes, Austin, (1994)).

It is yet another object of the present invention to provide an automated cell processor, a highly efficient means for producing mass quantities (over 100 billion in one run) of viable, sterile, genetically normal as well as functional myogenic cells whether genetically transduced or cytocline-converted. The cell processor has an intake system which will hold biopsies of various human tissues. The cell processor's computer will be programmed to process tissue(s), and will control time, space, proportions of culture constituents and apparatus functions. Cell conditions can be monitored at any time during the process. The output system provides a supply of cells suitable for transfer in cell therapy or for shipment (Figs. 14 to 15).

It is yet another embodiment in which myoblasts, and/or their physical, genetic, chemical derivatives, are used to treat cancer. Figs. 16 to 18 illustrate melanoma cancer cell death upon exposure to myoblasts in fusion medium. According to *Cancer Prevention and Control* edited by Greenwald, P., Kramer, B.S., and Weed, D.L. (Marcel Dekker, Inc. New York, 1995), skeletal muscles appear to be devoid of cancer, though malignant tumor and

metastases are found in every other organ. The very few cases of sarcoma reported are rare exceptions.

5 The recent immunocytochemical demonstration of dystrophin in DMD muscles 9 months after MTT indicated long term correction of genetic defect(s) can result from myoblast therapy (Fig. 19). This principle can apply to treat malfunctional insulin resistant muscles in Type II diabetes mellitus. As a universal gene transfer vehicle, donor myoblasts insert the whole normal genome and this 10 can repair any malfunction of skeletal muscle cells, rendering them insulin sensitive (Fig. 20).

15 Additional features and advantages are described in and will be apparent from the detailed description of the presently preferred embodiments and from the drawings. Further, all references described herein are incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Fig. 1 is a diagram of some of the known protein defects in DMD muscle cells that differ from normal muscle cells. These include membrane structural proteins that are decreased or absent in DMD such as dystrophin (DIN), dystrophin-related-protein (DRP) and dystrophin-associated-glycoproteins (DAG), enzymes elevated in serum levels of DMD patients such as creatinine phosphokinase (CPK), aldolase (ALD) and aspartate transaminase (AST), and mitochondrial (Mito) 25 protein differences. Although these protein defects are caused primarily or secondarily by the dystrophin gene defect, their correction will require multiple gene (G1 to G7) transfer.

30 Fig. 2 illustrates MHC-negative myoblasts and MHC-positive myoblasts. (A) represents human myoblasts assayed with anti-MHC class I antibodies and viewed under 35 fluorescent microscopy. The arrow indicates the MHC-negative myoblast. (B) is of the same slide viewed

under a regular light microscope. The arrow indicates the MHC-negative myoblast. Bar = 20 um.

Fig. 3 illustrates an analysis of myoblasts by cytofluorometry. Control: myoblasts reacted without anti-MHC class I antibodies. Samples: myoblasts reacted with anti-MHC class I antibodies.

Fig. 4 is a scatter diagram of the separation of the myoblasts that were negative for MHC-I antigen expression by cytofluorometry.

Fig. 5 illustrates fluorescent intensities of two groups of myoblasts after separation by cytofluorometry. (A) represents MHC-positive myoblasts. (B) represents MHC-negative or weakly expressed myoblasts. (A) and (B) are of the same magnification and the pictures were similarly processed. Bar = 30um.

Fig. 6 illustrates the angle of injection that determines myoblast distribution. (A) Oblique myoblast injection delivers donor myoblasts to the greatest number and area of recipient muscle fibers with the least leakage, resulting in the formation of the most mosaic fibers. (B) Transverse injection delivers donor myoblasts to a large number of fibers, but covers a smaller area and is more likely to result in leakage from the injection. (C) Longitudinal injection results in donor myoblasts fusing with each other, with fewer mosaic fibers being formed. (D) Focal injection results in only a small area of a few recipient muscle fibers being injected with donor myoblasts.

Fig. 7 illustrates donor myoblast nuclei labeled with fluoro-gold (FG), and are present in host muscle at seven days after MTT. (A) Mosaic myofiber with donor and recipient nuclei (black arrow) and donor myotube with donor myonuclei (white arrow). (B) Mosaic myofibers with donor (white arrows) and recipient (black arrows) nuclei.

Fig. 8 illustrates distributions of donor myoblasts labeled with FG in host muscle. Even distribution of donor myoblasts can be achieved with oblique myoblast

injection (A,B), or donor nuclei may appear in patches (C,D, white arrows) which gradually show more abnormal nuclei and debris. (C) represents a transverse injection and (D) a longitudinal injection.

5 Fig. 9 illustrates the production of allophenic twins with the mechanism of allophene formation.

Fig. 10 illustrates three littermates: 1 normal, 1 allophenic, 1 dystrophic (top to bottom).

10 Fig. 11 illustrates physiological recordings of maximal isometric twitch and tetanus tensions at 80 Hz elicited from the soleus muscles of the normal, allophenic, and dystrophic littermates. The allophene recordings resemble the normal, rather than the dystrophic recordings.

15 Fig. 12 illustrates soleus cross-sections from allophenic mice demonstrating core fibers (arrows) characteristic of muscle from DMD carriers. Whereas most myofibers remain normal in appearance, some degenerative characteristics such as fiber splitting and central nucleation are apparent.

20 Fig. 13 further illustrates soleus cross-sections from allophenic mice demonstrating core fibers (arrows) characteristic of muscle from DMD carriers. Although most myofibers remain normal in appearance, some degenerative characteristics are visible such as fiber splitting and central nucleation.

25 Fig. 14 illustrates the general layout of an automated cell processor.

30 Fig. 15 illustrates the detailed design of culture and harvest automated actions.

35 Fig. 16 is a comparison of cancer cell growth media with myoblast growth media on cultured melanoma (CRL6322) cells. (A, C, E, G) low magnification; (B, D, F, H) high magnification. (A, B) Melanoma cells cultured in cancer cell growth media for 9 days appear healthy, as evidenced by their numbers, elongated shapes, and branching processes. (C, D) Similar amount of melanoma cells

seeded and cultured in myoblast growth media for 9 days are more numerous and differentiated. (E, F) After 14 days in cancer cell growth media, the melanoma cells, while numerous, have become spherical in shape and detached from the surface, indicating that the cells are dead (E). At higher magnification only a few healthy cells remain (F). (G, H) After 14 days in myoblast growth media, the melanoma cells still appear numerous and healthy.

Fig. 17 illustrates myoblasts and melanoma cells (2:1 concentration ratio) co-cultured in myoblast growth media. (A, C) low magnification; (B, D) high magnification. (A, B) After 9 days in culture myoblasts and melanoma cells are numerous and remain differentiated. (C, D) After 14 days in culture myoblasts dominate the culture, with melanoma cells still surviving.

Fig. 18 illustrates myoblasts and melanoma cells co-cultured in myoblast growth medium for 4 days and then in myoblast fusion medium. (A, C, E, G) low magnification; (B, D, F, H) high magnification. (A, B) Myoblasts and melanoma cells (1:1 concentration ratio) after 5 days in myoblast fusion medium. Many melanoma cells have become spherical and detached from the surface and are not surviving in this medium. Myoblasts remain healthy and are beginning to fuse. (C, D) Myoblasts and melanoma cells (1:1 concentration ratio) after 10 days in myoblast fusion medium show numerous dying cells. Only a few can survive in this medium for this long, and they are detached and dying (D). (E, F) Myoblasts and melanoma cells (1:1 concentration ratio) after 19 days in myoblast fusion media. The surviving myoblasts retain their spindle shape and align with each other. Melanoma cells have become spherical and detached. (G, H) Myoblasts and melanoma cells (3:1 concentration ratio) after 19 days in myoblast fusion medium. Myoblasts have begun to fuse, forming myotubes.

Fig. 19 is an immunocytochemical demonstration of dystrophin in human muscle. Sarcolemmal localization of dystrophin is shown in normal control muscle (A) but not in DMD muscle (B). (C, D) DMD biceps brachii muscles from a subject who received MTT in one biceps and placebo injections in the contralateral muscle 9 mo before biopsies. Since blinding continues until the end of the study, the designation of the MTT muscle cannot be revealed. However, only one muscle shows sarcolemmal localization of dystrophin. (B), (C), and (D) were intentionally over-exposed to show immuno-reactive background elements not associated with the sarcolemma.

Fig. 20 illustrates (a) normal skeletal muscle metabolizing glucose with insulin. (b) In Type II diabetes mellitus, the major sequela of insulin resistance is decreased muscle uptake of glucose. It is possible that the glucose transporter in muscle is abnormal. It is known that insulin-mediated stimulation of tyrosine kinase and autophosphorylation are impaired. These latter defects can be corrected by MTT by normal gene expression (from Metabolism 6:6, 1993).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compositions and methods described herein will be illustrated for the treatment of individuals having hereditary neuromuscular diseases. However, it is contemplated that other hosts and other disease states may be treated with the inventive compositions and methods.

A. CONTROLLED CELL FUSION

Myoblasts have the unique ability to fuse with other cells. With the use of normal myoblasts, a full complement of normal genes can be introduced into any genetically abnormal cells through cell fusion. For example, the genetically abnormal cell could be a liver cell, heart muscle cell, or even a brain cell. The idea is to introduce a full complement of normal genes into

abnormal cells and, therefore, treat the genetic disease at the gene level and not at the hormonal or biochemical level.

For treating genetic diseases that involve structural protein abnormalities rather than regulatory protein abnormalities, it would be useful to control, initiate or facilitate cell fusion once myoblasts were injected into the body. It is known that myoblasts fuse readily at low serum concentrations in culture. The process is more complex in the *in vivo* situation. As the myoblasts are injected intramuscularly into the extracellular matrix (ECM), injection trauma causes the release of basic fibroblast growth factor (bFGF) and large chondroitin-6-sulfate proteoglycan (LC6SP) (Young, H.E., et al., *J. Morph.*, 201:85-103 (1989)). These latter growth factors stimulate myoblasts proliferation. Unfortunately, they also stimulate the proliferation of fibroblasts that are already present in increased amounts in the dystrophic muscle. It is, therefore, necessary to inject as pure as possible fractions of myoblasts in MTT without contaminating fibroblasts.

Controlled cell fusion can be achieved by artificially increasing the local concentration of LC6SP over the endogenous level at the transfer site. In muscles, this is achieved by including approximately 5 μ M of LC6SP in the transfer medium. In addition, insulin facilitates the developmental process *in vitro*, and may result in the formation of myotubes soon after myoblast injections. The use of LC6SP (ranging from approximately 5 μ M to about 5mM) in the transfer medium will likely lead to greater MTT success.

B. MYOBLASTS: THE UNIVERSAL GENE TRANSFER VEHICLES
Whereas MTT results in the formation of genetic mosaicism with gene transfer occurring *in vivo*, the production of heterokaryons *in vitro* has immense medical application. This can be achieved by controlled cell fusion with myoblasts.

This research relates to the *in culturo* transfer of the normal nuclei with all of their normal genes from donor myoblasts into the genetically normal and/or abnormal cells, e.g. the cardiomyocytes. This development is especially important considering that cardiomyopathic symptoms develop in mid-adolescence in about 10% of the DMD population. By age 18, all DMD individuals develop cardiomyopathy. Undoubtedly, the ability to replenish degenerated and degenerating cardiomyocytes will have an immense impact on heart diseases even in the normal population where there is a great shortage of hearts for transplantation.

Normal cardiomyocytes have a very limited ability to proliferate *in vivo* or *in vitro*. The heart muscles damaged in heart attacks or in hereditary cardiomyopathy cannot repair themselves through regeneration. By integrating the skeletal muscle cell characteristic, mitosis, heterokaryotic cardiomyocytes will be able to proliferate *in vitro*.

Controlled cell fusion between normal myoblasts and normal cardiomyocytes may result in heterokaryons exhibiting the characteristics of both parental myogenic cell types. Clones can be selected based on their abilities to undergo mitosis *in vitro* to develop desmosomes, gap junctions, and to contract strongly in synchrony after cell transplantation.

These genetically superior cells can then be delivered through catheter pathways of the type described by Jackman WM, et al. (In: Zipes DP, and Jalife J, eds. *Cardiac electrophysiology. From Cell to Bedside*. Philadelphia: WB Saunders Company, 491-502, (1990)) after mapping of the injured sites. With the ability to grow large quantity of these cardiomyocytes, the correction of structural, electrical and contractile abnormalities in cardiomyopathy can be tested first in dystrophic, cardiomyopathic hamsters and then in humans.

The genetic transfer of the mitotic property of myoblasts onto cardiomyocytes with *in vitro* controlled cell fusion enables the resulting heterokaryotic cardiomyocytes to multiply, yielding enough numbers of cells for the cell transplant to be effective.

Recently, it was reported that fetal mouse cardiomyocytes grafted into the myocardium of syngeneic hosts formed nascent intercalated disks between host and donor cells (Soonpaa MH, et al., *Science*, 264:98-(1994)). The use of fetal cells for cell transplant has and will continue to raise ethical questions. The fact still remains that fetal cells will not produce enough cardiomyocytes to mend a myocardial infarct. The bioengineering of mitotic cardiomyocytes using myoblasts provides a solution to the problem in view of reported studies that recombinant genes introduced into cardiomyocytes are expressed for at least 6 months, and appear to be regulated normally by humoral signals.

Whereas myoblast transfer into the dystrophic myocardium followed by *in vivo* controlled cell fusion may provide a structural impediment at the infarct, it remains to be shown that the myoblasts will integrate well with the cardiomyocytes, considering that the pumping action of the heart will disaggregate the developing cells from the host myocardium.

C. COSMETIC USAGE

In a broader sense, the cell therapy concept can significantly contribute to the field of plastic surgery. With cell therapy, implantation of silicone could be avoided. The use of myoblasts and/or fat cells could be used in a much more natural way to replace silicone injections for facial, breast and hip augmentation. Modified adipose tissue involving mixing and/or hybridization of myoblasts and fat cells can be used to control size, shape and consistency of body parts. Since muscle cells do not break down as easily as fat cells, good results may be long-lasting. Today, body builders

are in search of increasing muscle mass and function at the right places. The use of myoblast transfer to boost muscle mass is a natural solution.

D. SUPERIOR CELL LINES

5 The establishment of superior cell lines of myoblasts is a high-risk challenge, but its benefits are numerous. These cell lines should be highly myogenic, nontumorigenic, nonantigenic, and will develop very strong muscles.

10 A unique property of myoblasts is their loss of major histocompatibility complex class I (MHC-I) surface antigens soon after they fuse. This has important implications in the usage of an immunosuppressant after myoblast transfer therapy. (See Huard J, et al., *Muscle Nerve*, 17:224-34 (1994); Roy R, et al., *Transpl Proc*, 25:995-7 (1993); and Huard J, et al. *Transpl Proc*, 24:3049-51 (1992)). The immunosuppression period depends on how soon the myoblasts lose their MHC-I antigens after MTT. Even more ideal is the establishment of a myoblast cell line in which MHC-I antigens are absent, thereby allowing MTT without immunosuppression.

15 In our study, human myoblasts were cultured from normal muscle biopsies in accordance with the methods disclosed in Law, P.K., et al., *Cell Transplantation*, 1:235 (1992) and Law, P.K., et al., *Cell Transplantation*, 2:485 (1993). The MHC-I antigens expressed on the myoblasts were demonstrated with fluorescent immunoassay. Cell cycle synchronization of myoblasts was carried out by adding colchicine in the growth medium and incubating for 48 hours. The myoblast preparations used in the experiment were 98% pure as assessed by immunostaining with the monoclonal antibody (MAb) anti-Leu-19.

20 Myoblasts were incubated with anti-MHC-I MAb (mouse 1:25 dilution, Silenus Lab, Australia) at room temperature. After washing, the myoblasts were incubated with FITC conjugated anti-mouse-IgG (Sigma) for 45 minutes and examined under fluorescence microscope with

- wide band ultraviolet (UV) excitation filter. Cytofluorometry was performed with a Becton-Dickinson cell sorter operated at 488 mM. Myoblast control was carried out by omitting the first antibodies in the immunoassay as the background of autofluorescence.
- 5 91.7% of the myoblasts reacted with MHC-I MAb. The reactions ranged from strong to weak. The remaining 8.3% of the myoblasts were negative for MHC-I antigen expression. Fig. 2 illustrates both MHC-negative myoblasts and MHC-positive myoblasts. The MHC-negative myoblasts were successfully separated by cytofluorometry, which is illustrated in Figs. 3,4. Both groups of myoblasts were then cultured for three weeks without significant difference in proliferation.
- 10 The lack of MHC-I antigens on these myoblasts may enhance survival of these myoblasts in recipients after MTT. Fig. 5 illustrates the fluorescent intensities of both MHC-positive myoblasts and MHC-negative or weakly expressed myoblasts after separation by cytofluorometry.
- 15 The immunosuppressant, cyclosporine, has many side effects and by suppressing the immune system, allows infection to prevail. Myoblasts without MHC-I antigen expression may contribute to a new cell line more capable of surviving in the host than the regular myoblasts.
- 20 The superior cell line will eliminate the need to use the immunosuppressant, and will provide a ready access for patients who do not have a donor.
- 25 This superior cell lines have to be derived from clones of primary myoblast cultures because they are selected for their unique properties. Unfortunately, it has been shown that all clones of myoblasts eventually produce tumors if allowed to proliferate excessively. Thus, these cell lines should not be allowed to proliferate over 30 generations.
- 30 **E. MYOBLAST INJECTION METHODS**
- 35 Aside from donor cell survival in an immunologically hostile host, cell fusion is the key to strengthening

dystrophic muscles with MTT. To improve the fusion rate between host and donor cells, various injection methods aimed at wide dissemination of donor myoblasts were tested and compared. These included injecting diagonally through the myofibers, perpendicular to the myofiber surface, parallel to the myofibers, and at a single site into the muscle. Fig. 6 illustrates myoblast distribution as a function of the angle of the injection. The goal was to achieve maximum cell fusion with the least number of injections.

Fluoro-gold (FG, 0.01%) labeled human or mouse (C57BL/6J-gpi-1c/c) myoblasts (0.05 ml of a 10^5 cells/ml solution) were injected into the gastrocnemius muscles of twenty normal 3-month old normal mice (C57BL/6J-gpi-1b/b). Host mice were immunosuppressed with a daily subcutaneous injection of cyclosporine at 50 mg/kg body weight. Groups of mice were sacrificed on day 7, 14, 24, 34, and 44 after cell injection. Transverse sections of injected muscles were examined with fluorescence microscopy. The cell fusion rates were estimated by calculating the percentage of host muscle fibers bearing donor nuclei out of the total number of muscle fibers in the area of donor cell covered. The glucose phosphate isomerases (GPI) of the injected muscles were also examined with agarose gel electrophoresis (200 V anode to cathode, 3 hours, pH 8.6). The first appearance of mosaic myofibers in the tissue sections was within seven days after cell injection. This is illustrated in Fig. 7. The highest fusion rate achieved was 72.2%. The electrophoreograms of GPI showed host donor and mosaic GPI in muscle specimens at least up to 44 days after MTT. Myoblasts injected obliquely through the myofibers were widely and evenly distributed with ejection of the myoblasts as the needle is withdrawn. This is shown in Fig. 8. Myoblasts injected perpendicular to the myofibers were partially distributed, while myoblasts that were injected

longitudinally through the core of the muscles and parallel to the myofibers were poorly distributed. Similarly, injection at one spot gave poor distribution and fusion. Considering that a small volume of a concentrated solution causes less muscle damage than a larger volume of a relatively less concentrated solution, and in view of the trauma caused by injection decreases the regenerative capability of dystrophic muscles, the technique of myoblast delivery is essential for MTT success.

Although oblique injection has been used in our clinical trials, there is room for improvement since human muscles are larger and the myofiber orientation of different muscle groups have to be well-studied by the orthopedic surgeons who administer myoblast injections. Judging from previous mouse studies, 20% normal myonuclei were able to maintain normal phenotype in dystrophic myofibers.

F. EXERCISE AND PHYSICAL THERAPY

Strenuous exercise causes damage to dystrophic myofibers. Lack of dystrophin causes the vulnerable sarcolemma to tear upon contraction. Other cell types are somewhat spared from degeneration because they do not contract. Thus, body building is counter-productive in DMD patients to compensate for loss of muscle mass and strength.

The use of exercise, however, in relation to MTT has not been studied. In dystrophic animals, it is well known that exercise hastens the degeneration of myofibers and thus aggravates the dystrophic condition, that is with dystrophic muscle fibers alone. The situation is different from MTT in which an attempt is made to produce a mosaic muscle containing normal, mosaic, and dystrophic fibers. The essence of MTT is to reconstruct the genetics and improve the phenotypes of dystrophic muscles. Thus, intensive exercise may induce the release of host satellite cells that will fuse with normal myoblasts to

produce mosaic fibers. Undoubtedly, such dystrophic degeneration will induce normal muscle regeneration. Implanted myoblasts not only fuse to the newly sealed regions of damaged myofibers, but also survive as satellite cells. Mild exercise done shortly after MTT can be designed to facilitate myoblast mixing, alignment, and fusion, and to provide physical therapy to the newly formed fibers. Moderate exercise after innervation of newly formed fibers is likely to enhance the development of normal and mosaic fibers. Disuse plays a major role in the continued deterioration of dystrophic muscles, and physical therapy is prescribed for dystrophic patients. Disuse or lack of cross-bridge interaction results in a decrease of calcium binding. As a result, the excessive intracellular calcium promotes muscle damage in dystrophic muscles.

G. MYOTUBE TRANSFER

In the later stages of DMD, there remains fewer myofibers to be repaired with MTT. Formation of new fibers to replenish degenerated cells is further complicated by the presence of excessive connective and fat tissues. While it takes approximately 1 to 3 weeks for donor myonuclei to be incorporated into dystrophic fibers for repair, it takes over 4 months for donor myoblasts to develop into mature normal fibers *de novo* to replenish lost cells. Meanwhile, the impediment to developing myotubes to be vascularized, innervated, and connected to tendons all threaten their survival. Enough nutrients have to be present for the developing fibers to lay down the contractile filaments myosin and actin. Neither electrical nor contractile activity is normal for the development of the fibers. This is the time when myotube transfer may be of help.

Transplants of newborn normal muscles or myotubes into dy^{2J}dy^{2J} dystrophic mouse muscles have been shown by this inventor to produce normal muscle function and structure. (See Law, P.K. and Yap, J.L., *Muscle Nerve*,

2:356-63 (1979)). Myotubes are easily obtained in culture through natural myoblast fusion by exposing confluent cultures to the fusion medium. In fact, small muscles have been produced with spontaneously contracting fibers in culture. The young fibers exhibit sarcomeres and immunostain positively for myosin.

5 Myotube transfer can be administered through injection with larger gauge needles. Better still, they can be surgically implanted into the beds of fat and connective tissues dissected and removed by surgeons. 10 Since muscles can develop great forces and scar tissues are inert, the developing muscles will force the scar tissues aside throughout their existence.

15 Myotube transfer provides bioengineered young fibers *in vitro*. These fibers have lost their MHC-I surface antigens and are thus nonantigenic. Myotube transfer will not need to be administered with cyclosporin. For patients previously infected with cytomegalovirus (CMV) or other viruses, myotube transfer will be the choice.

20 In addition, for autosomal dominant diseases such as facioscapulohumeral dystrophy (FSH), myotonia congenita, myotonia dystrophica and certain forms of congenital muscular dystrophy and limb-girdle dystrophy, formation of mosaic fibers may not be useful since nuclear 25 complementation may not be effective. The use of entirely normal myotubes through myotube transfer will undoubtedly open new avenues for treatment.

H. ALLOPHENIC MICE

30 Allophenic mice or mouse chimaeras are mice mosaics with two or more genotypes. They are produced by blastomere recombination (see Hogan B., et al. Manipulating the Mouse Embryo. A Laboratory Manual. Cold Spring Harbor Laboratory, (1986)) or by the 35 artificial aggregation of embryos from two different strains of mice. In addition to being important specimens to study the clonal origins of somites and their muscle derivatives, allophenic mice have been shown

by this inventor and others to demonstrate dystrophy suppression in natural development when genetically normal and dystrophic myogenesis coexist.

5 By aggregating half embryos of normal (129 strain) and dystrophic (C57BL/6J dy^{2J}dy^{2J} strain) mice as shown in the diagram of Fig. 9, sets of allophenic twins were produced consisting of chimaeric mice and their normal and dystrophic littermates (Fig. 10). Although the dystrophic gene was present in the muscle fibers
10 according to genotype marker analyses, these allophenic mice showed normal behavior, life span, and essentially normal muscle function shown in Fig. 11 and structure in Figs. 12 and 13.

15 Muscle fibers of these allophenic mice highly resemble those of the Duchenne female carriers. Whereas the dystrophic soleus contains 70% or more degenerating fibers, only 3 to 5% of the allophenic soleus fibers are abnormal. Many of these abnormal fibers showed "cores" as seen in the Duchenne carriers. Through natural cell fusion, normal myoblasts fuse with dystrophic ones to form mosaic myotubes that develop into phenotypically normal fibers.
20

I. NORMAL CHILD FOR A DUCHENNE CARRIER

25 Conceivably the technology of *in vitro* fertilization and blastomere recombination used in the allophenic mouse studies can be applied to human. Known carriers may thus have better chances of bearing normal children.

30 Accordingly, ova from a carrier and from a normal female can be obtained and fertilized *in vitro* with sperm recovered from the carrier's husband. The fertilized egg of the carrier has a 50/50 chance of being normal or dystrophic. Regardless of its genotype, its mixing with the normal fertilized egg will ensure the development of a normal phenotype. After culturing the embryo into the blastocyst stage, it can be implanted into the uterus of the carrier. The latter can be induced to be
35

pseudo-pregnant with human gonadotrophin, thus allowing easy implantation.

5 The use of in vitro fertilization protects the mother. Abnormal developing embryos after blastomere recombination can be discarded. Furthermore, no blastomere needs to be removed for genetic analysis.

10 Since the fertilized egg of the carrier has 50% chance of being normal, a PCR analysis for dystrophin messenger RNA can be conducted on blastomeres removed from the embryo at the blastocyst stage. Unfortunately, 15 this risks damaging the embryo by removing part of it at an early developmental stage.

J. AUTOMATED CELL PROCESSORS

15 With the great demand for normal myoblasts, myotubes and young muscles, the labor intensiveness and high cost of cell culturing, harvesting and packaging, and the fallibility of human imprecision, an automated cell processor is needed. Such a processor would be capable 20 of producing mass quantities, over 100 billion per run, of viable, sterile, genetically well-defined and functionally demonstrated biologics, for example, myogenic cells.

25 The automated cell processor will be one of the most important offspring of modern day computer science, mechanical engineering and cytogenetics (Fig. 14). The intakes will be for the biopsies of various human tissues. The computer will be programmed to process tissue(s), with precision control in time, space, and proportions of culture ingredients and apparatus 30 maneuvers. Cell conditions can be monitored at any time during the process, and flexibility is built-in to allow changes. Different protocols can be programmed into the software for culturing, controlled cell fusion, harvesting and packaging. The outputs will supply cells, 35 which will be ready for shipment or for injection using cell therapy. The automated cell processor will be self-contained in a sterile enclosure large enough to

house the hardware in which cells are cultured and manipulated (Fig. 15).

5 This inventor has developed a transfer medium that can sustain the survival and myogenicity of packaged myoblasts for up to 3 days at room temperature. Survival up to 7 days can be achieved when the myoblasts are refrigerated. This will allow the cell packages to be delivered to remote points of utilization around the world.

10 The automated cell processor will simply replace current bulky inefficient culture equipment and elaborate manpower. Its contribution to human healthcare will undoubtedly be significant, and the manufacturing costs are expected to be relatively low.

15 K. CELL BANKS

The automated cell processors will constitute only a part of cell banks. Ideally, donor muscle biopsies can be obtained from young adults aged 8 to 22 to feed the inputs of the automated cell processor. This will depend 20 on the availability of healthy volunteer donors. Each donor has to undergo a battery of tests that are time-consuming and expensive. Based on the test results and the donor's physical condition, one can determine if the donor cells are genetically defective or infected 25 with viruses and/or bacteria. These are the advantages of biopsies of mature tissues from adults. The major disadvantage, however, is that mature cells often do not divide, and even if they do, there is a limited number of generations that can propagate before becoming 30 tumorigenic or nonmitotic.

Human fetal tissues can potentially provide unlimited supplies of dividing cells. However, aside from ethical issues, it is difficult to determine the genetic normality of these cells, notwithstanding the 35 existence of polymerase chain reaction (PCR) which is used to screen many human genetic diseases.

As for the muscular dystrophies, the use of muscle primordia of fetal calves derived from *in vitro* fertilization of genetically well-defined background may be an alternative. Sperms and ova can be recovered from inbred strains of cattles that are known for their muscle strength and mass. *In vitro* fertilization will be followed by embryo culture and implantation into the uteruses of pseudo-pregnant cows. The fetuses are removed by Sicilian sections at specific developmental stages of the embryos. The muscle primordia that are rich in myoblasts can then be dissected out to feed into the automated cell processors.

Transplantation of cattle cells into humans constitutes xenografting. Due to the significant differences between the human and the cattle immune systems, these xenografts will likely survive, develop and function in the recipients without the need for immunosuppressants. However, the method will be tested with and without immunosuppressants.

L. MYOBLAST DERIVATIVES VS. CANCER
Evolution is one continual experiment through ages with numerous statistics. The near absence of cancer metastases in skeletal muscles suggests that the physical, electrical, mechanical or chemical presence of myogenic cells and derivatives prevents or annihilates cancer.

In our study we showed that the physical and biochemical conditions of myoblast at the cell fusion stage caused the death of melanoma cancer cells (Fig. 16 to 18). This does not preclude the potential effect of similar or different conditions, including electrical and mechanical, of other myogenic cells at different developmental stages.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without

departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

I CLAIM:

1. A method of treating mammalian disease conditions that are hereditary, degenerative, debilitating, fatal or undesirable, comprising the steps of:
 - 5 culturing a supply of myogenic cells that are normal, genetically transduced or phenotypically converted, the myogenic cells comprising myoblasts, myotubes, young muscle fibers and converted cell types;
 - 10 administering a therapeutically effective dosage of an immunosuppressant to the host; and thereafter selecting and administering from said supply a therapeutically effective dose of myogenic cells or cells converted from myogenic cells to the host,
 - 15 whereby tissue/organ size, shape and/or function are improved and/or the disease condition(s) are prevented, alleviated or annihilated.
- 20 2. The method of claim 1 in which treatment of the disease condition(s) is not limited to the usage of the mechanical electrical or physical properties of said myogenic cells, but includes the usage of biochemicals secreted/released.
- 25 3. The method of claim 1 in which myoblast therapy is used to treat neuromuscular diseases, cancer, Type II diabetes mellitus, cardiomyopathy, bone/cartilage degeneration, hemophilia B, anemia, human growth hormone deficiency and any other hereditary, degenerative, debilitating, fatal and /or undesirable disease conditions or malfunction.
- 30 4. The method of claim 1 wherein the converted cell types include but are not limited to osteoblasts and chondrocytes.

5. A method for controlling cell fusion, comprising the step of:

increasing the concentration of large chondroitin-6-sulfate proteoglycan in an amount
5 sufficient to exceed the endogenous level of large chondroitin-6-sulfate proteoglycan.

6. The method of claim 5 wherein the concentration of large chondroitin-6-sulfate proteoglycan is increased in
10 a cell culture medium.

7. The method of claim 5 wherein the concentration of large chondroitin-6-sulfate proteoglycan is increased in
15 a medium for transfer into a patient or host.

8. The method of claim 5 wherein the concentration of large chondroitin-6-sulfate proteoglycan is approximately

9. A method for controlling the size, shape or
20 consistency of human body parts, comprising the steps of:
culturing billions or more myoblasts from mammals;
and

25 reintroducing the myoblasts and/or their physical,
genetic cell derivatives into the mammal body parts to
augment the size, shape, consistency, structure,
biochemistry and/or function.

10. The method of claim 9, further including the steps of
mixing myoblasts and cultured fat cells in appropriate
30 proportions and transferring the mixture into one or more
body parts to produce the desired size, shape, or
consistency, and/or function.

11. A cloned cell line derived from a culture of
35 genetically normal human or animal myoblasts, said cloned
cell line being characterized as lacking or mildly
expressing MHC-I antigens.

12. A cloned cell line derived from a culture of myogenic cells which are genetically/phenotypically altered or have been transduced with a foreign gene, or are heterokaryons resulted from controlled cell fusion, the cloned cell line being characterized as lacking or 5 mildly expressing MHC-I antigens.

13. The use of the cloned cell lines in Claims 11 and 12 in cell transplantation to treat debilitating, fatal, 10 hereditary degenerative or undesirable conditions of mammals including human.

14. An automated cell processor capable of producing at a single time enormously large quantities of functional myogenic cells, normal or transformed, comprising:
15 an intake system for processing biopsies of human or animal tissues/cells;
 a means for controlling the time for processing the tissues;
20 a means for controlling the composition of the culture medium for maintaining cell growth;
 a means for controlling the growing conditions for the cells;
 a means for culturing the cells;
25 a means for controlling cell fusion;
 a means for harvesting the cells; and
 a means for packaging the cells.

15. The automated cell processor of claim 14 wherein the 30 quantity of the myogenic cells is approximately greater than 100 billion.

16. A method for producing a genetically normal child from a Duchenne female carrier, comprising the steps of:
35 fertilizing in vitro an ovum of a Duchenne carrier with a sperm of her choice;

fertilizing *in vitro* an ovum of a genetically normal female with a sperm of the same man;

blastomere recombination of the two fertilized eggs;

culturing the resultant embryo into the blastocyst
5 stage; and

implanting the blastocyst into the uterus of the Duchenne carrier rendered pseudopregnant with human gonadotropin.

10 17. A method for producing cardiomyocytes capable of proliferation, comprising the steps of:

mixing cardiomyocytes and normal myoblasts *in cultura* in amounts and proportions sufficient to achieve natural or controlled cell fusion; and

15 selecting and then cloning cardiomyocytes that are capable of proliferation.

18. The method of claim 17, including the step of transferring the proliferating cardiomyocytes into a
20 patient or host to treat cardiomyopathy or other heart diseases.

19. The method of claim 1 wherein the myogenic cells comprise skeletal, smooth, and cardiac cells in origin.

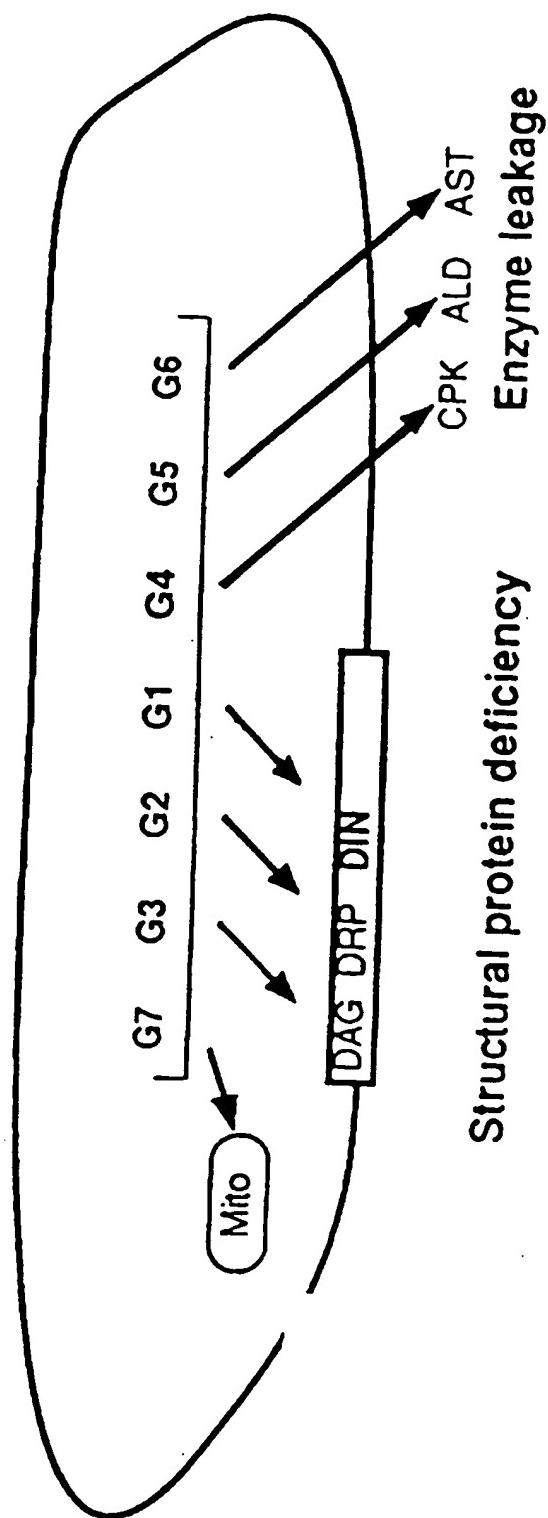


FIG. I

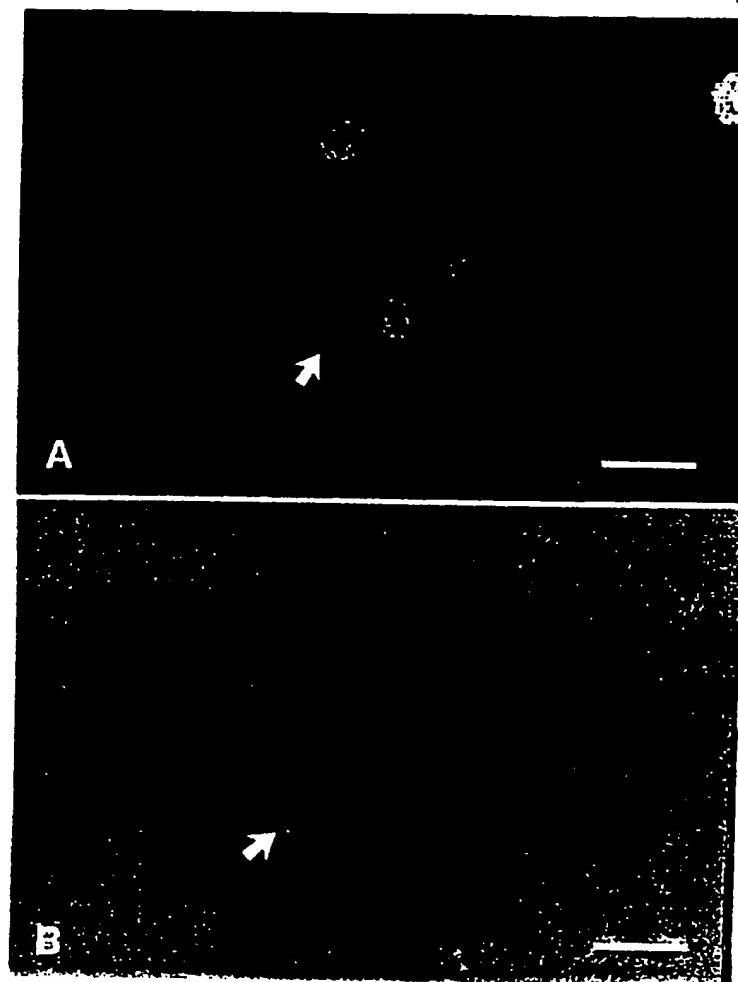


FIG. 2

3/20

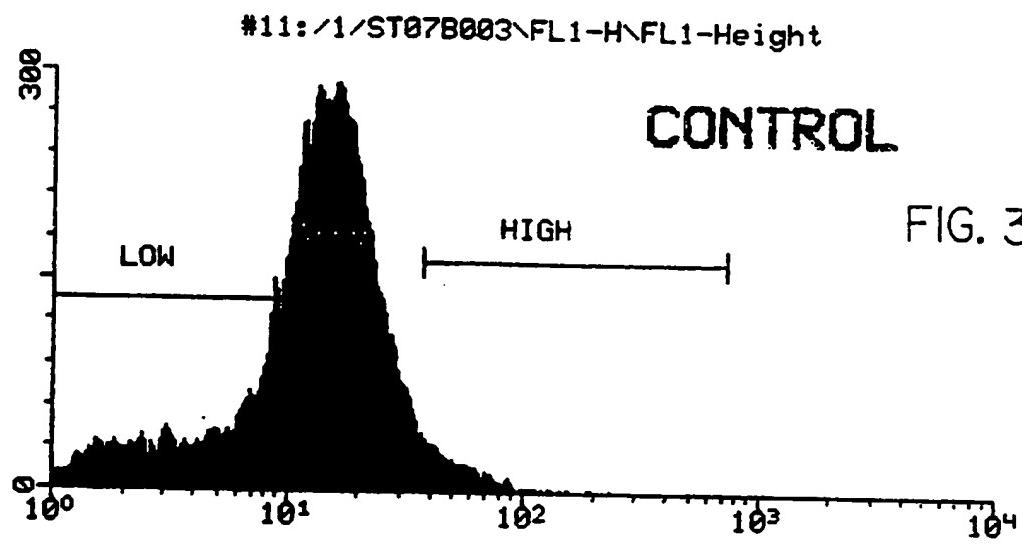
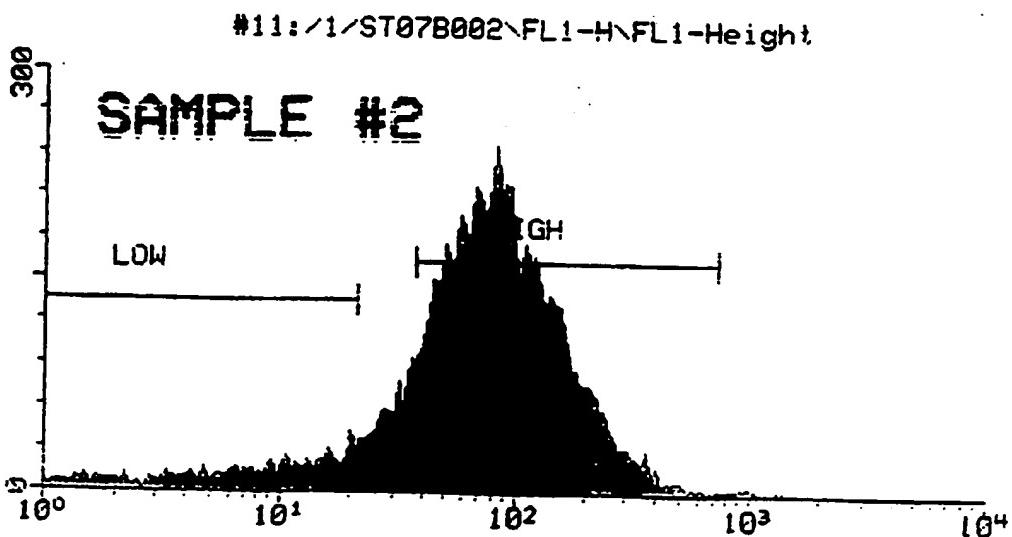
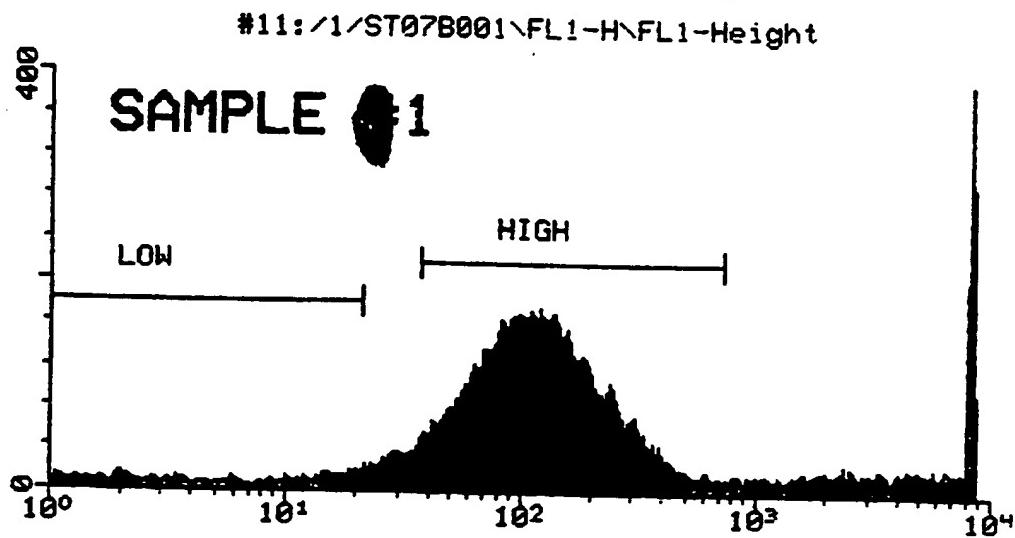


FIG. 3



4/20

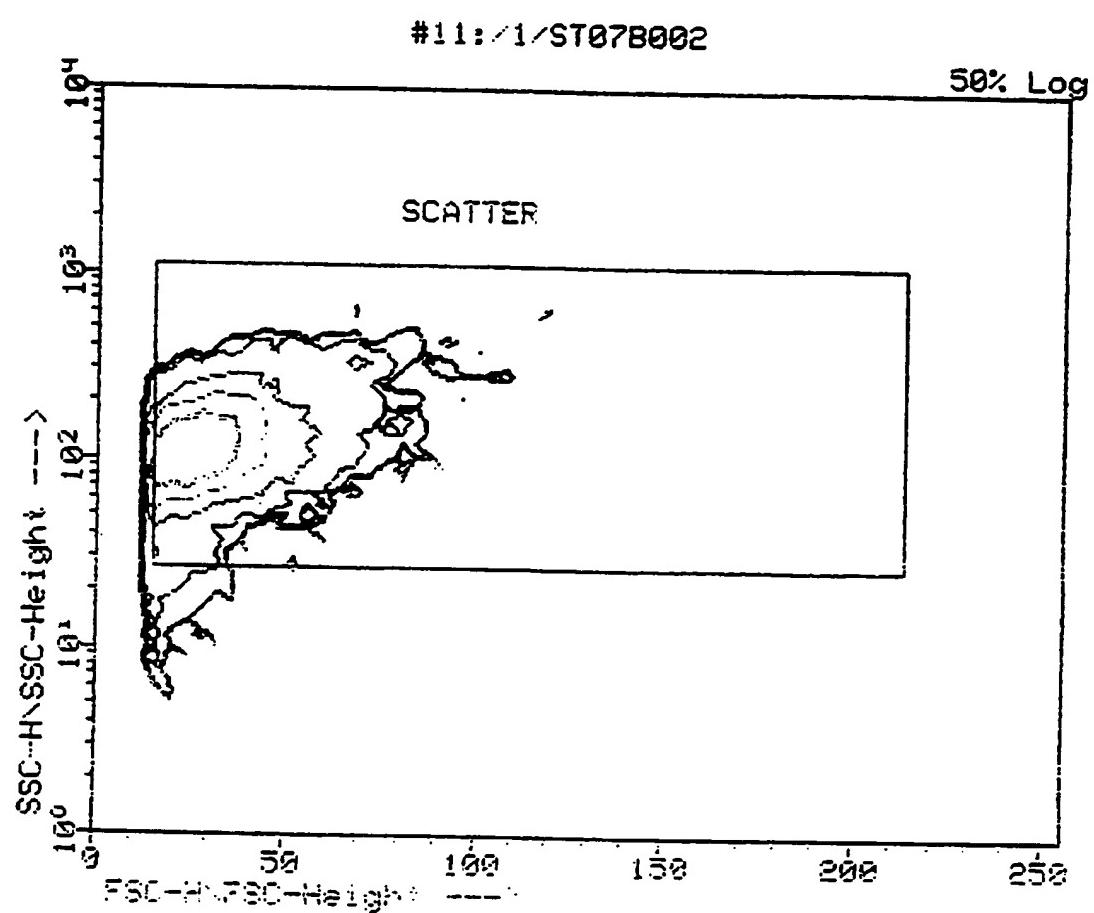


FIG. 4

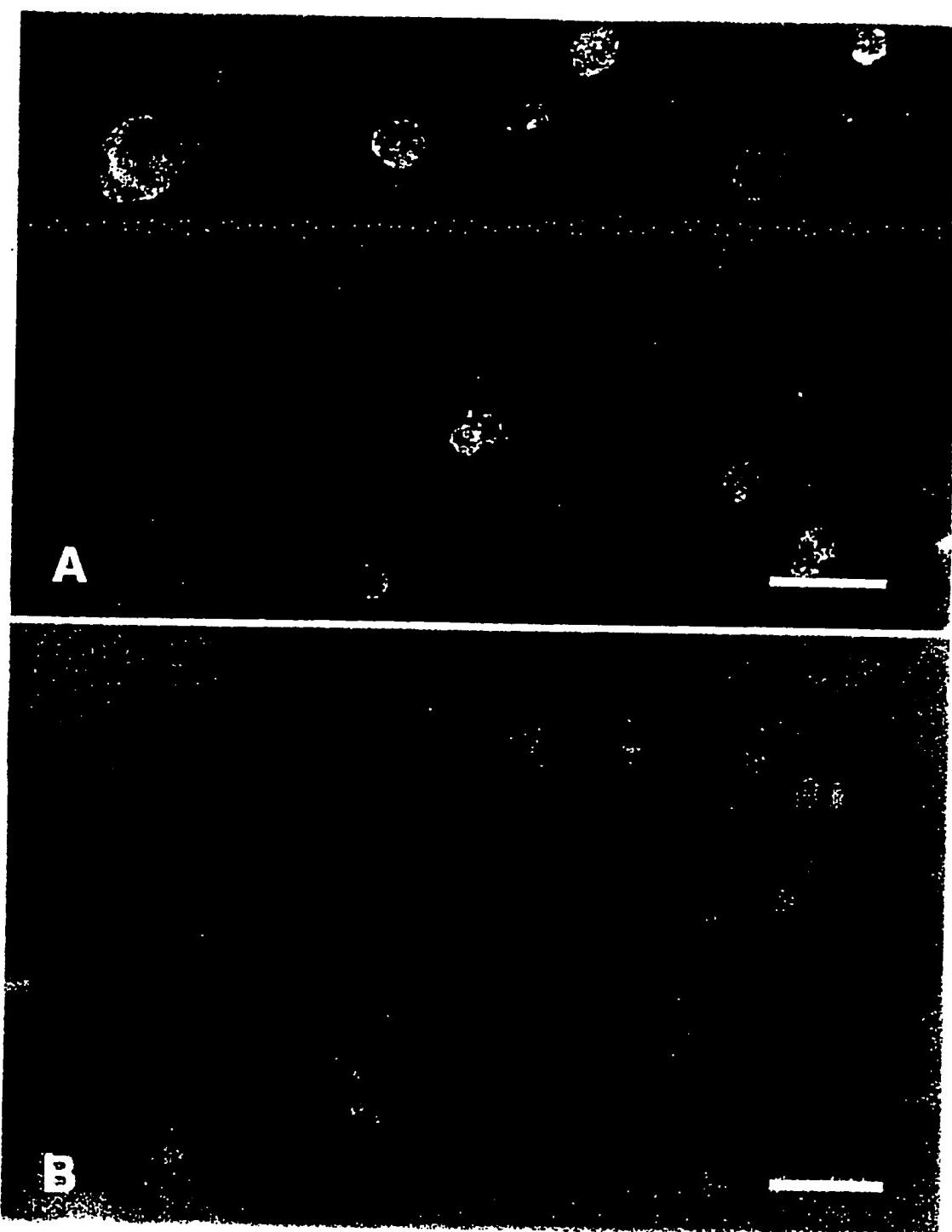


FIG. 5

ANGLE OF INJECTION DETERMINES
MYOBLAST DISTRIBUTION A>B>C>D

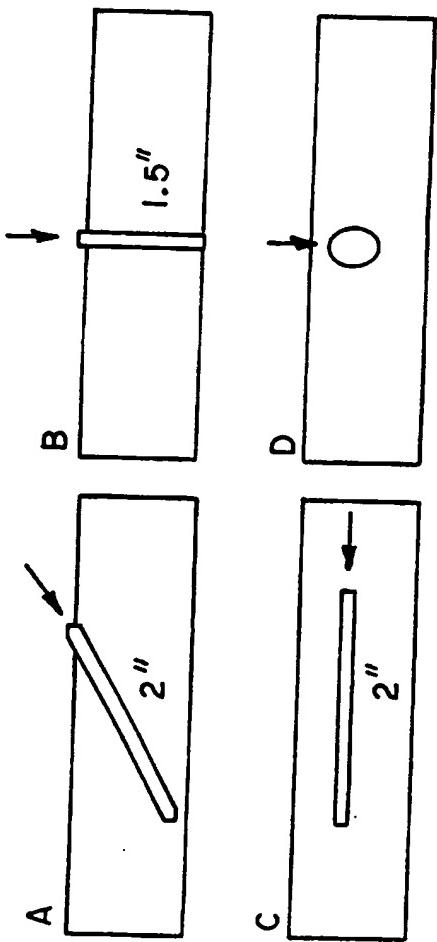


FIG. 6

W 96/18303

PCT/US95/16093

7/20

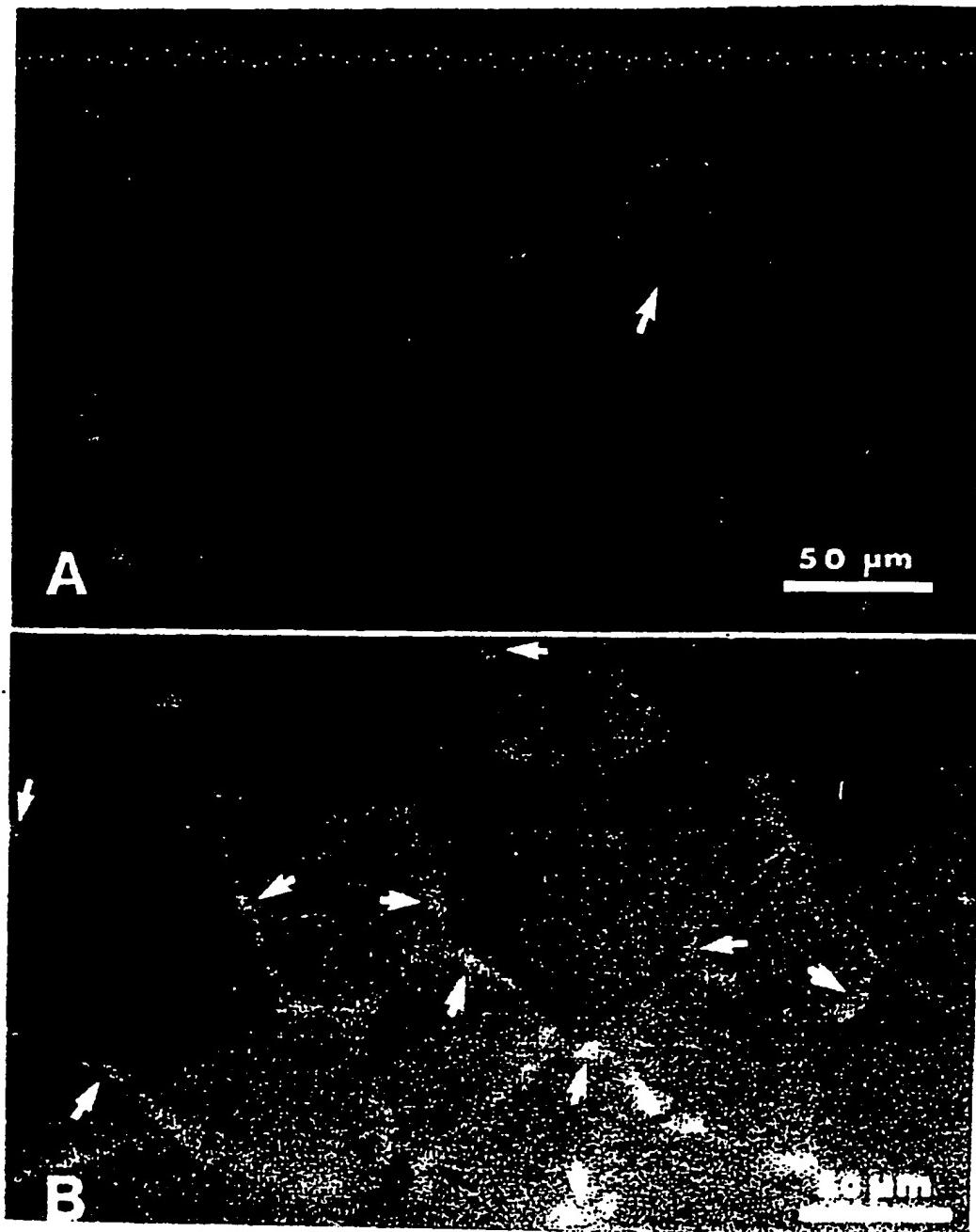
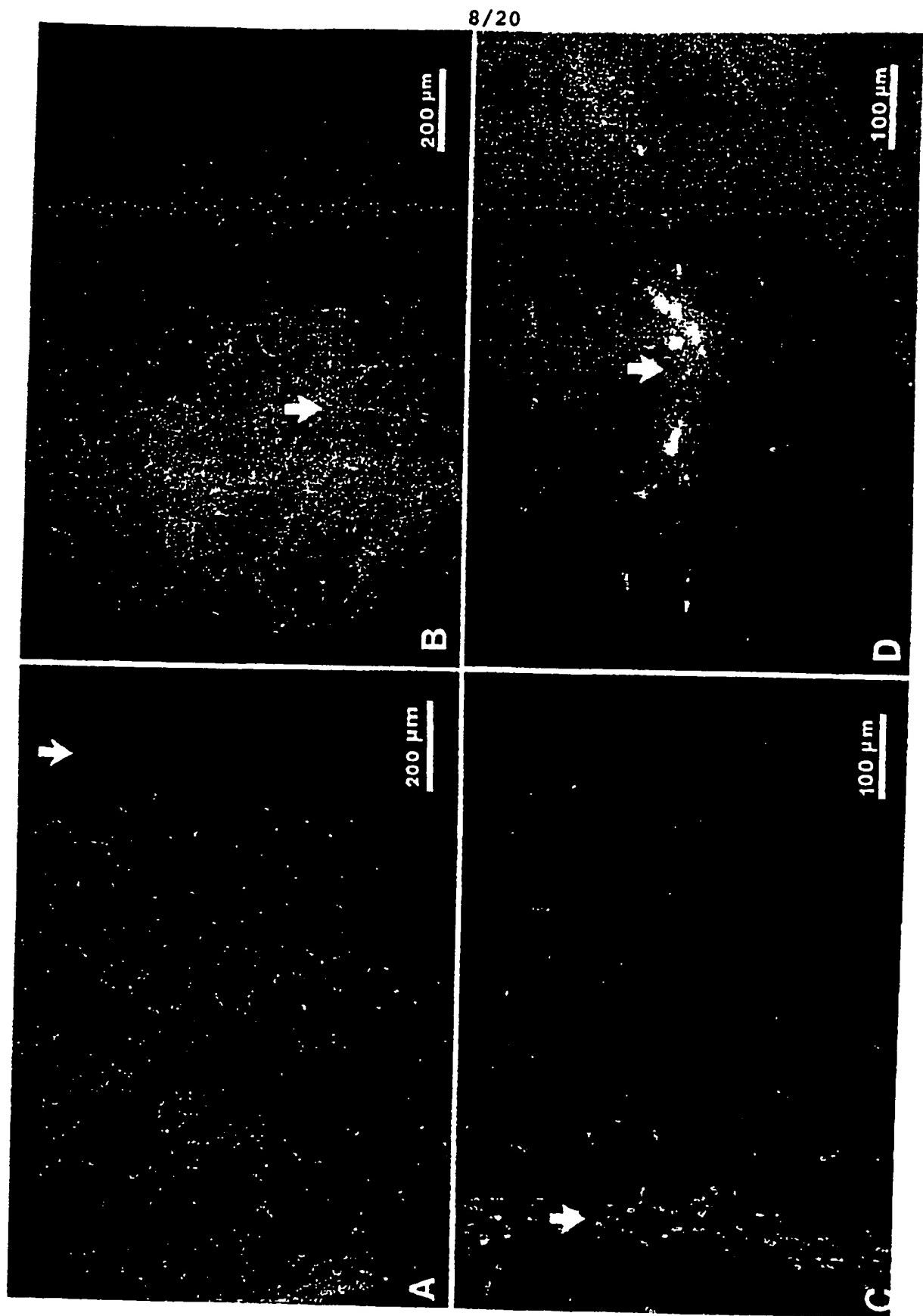


FIG. 7



9/20

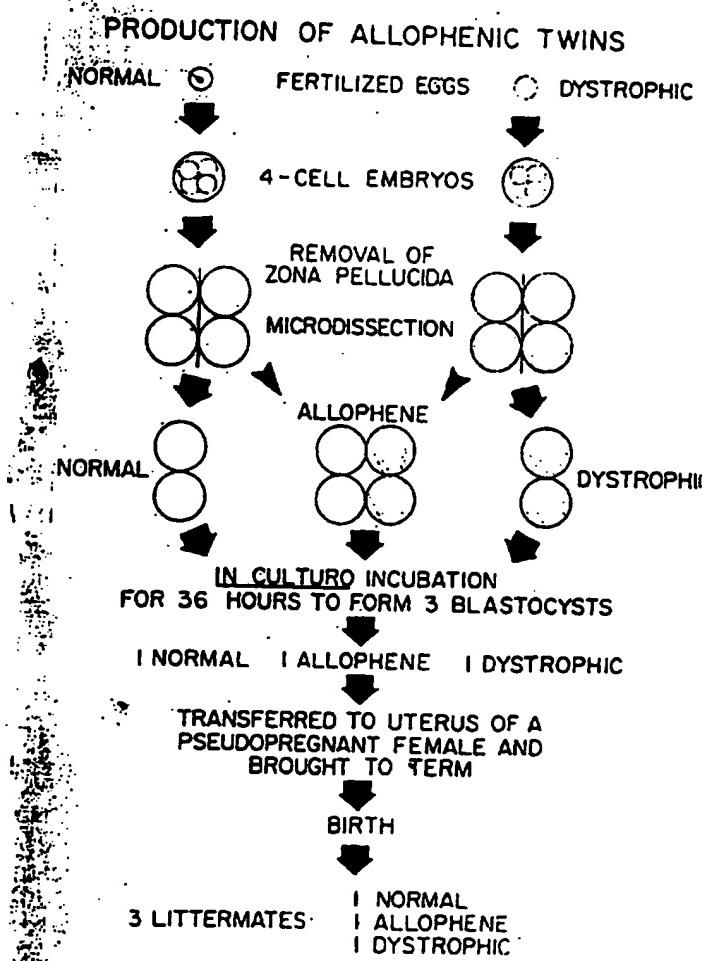


FIG.9



FIG. 10

11/20

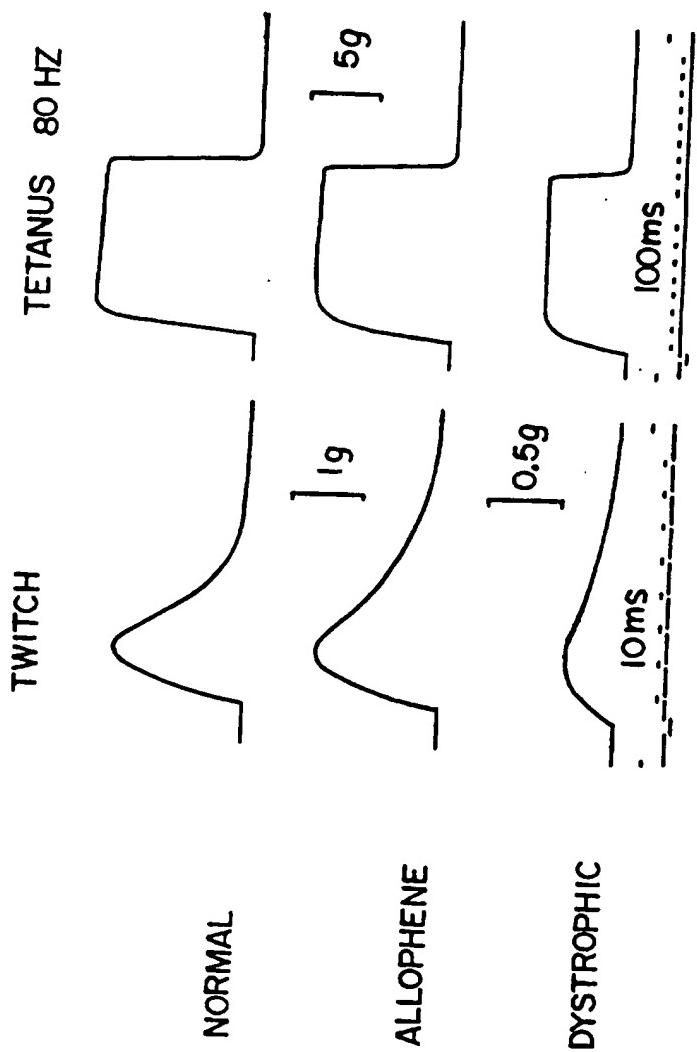


FIG. II



FIG. 12



FIG. 13

14/20

Instrument components

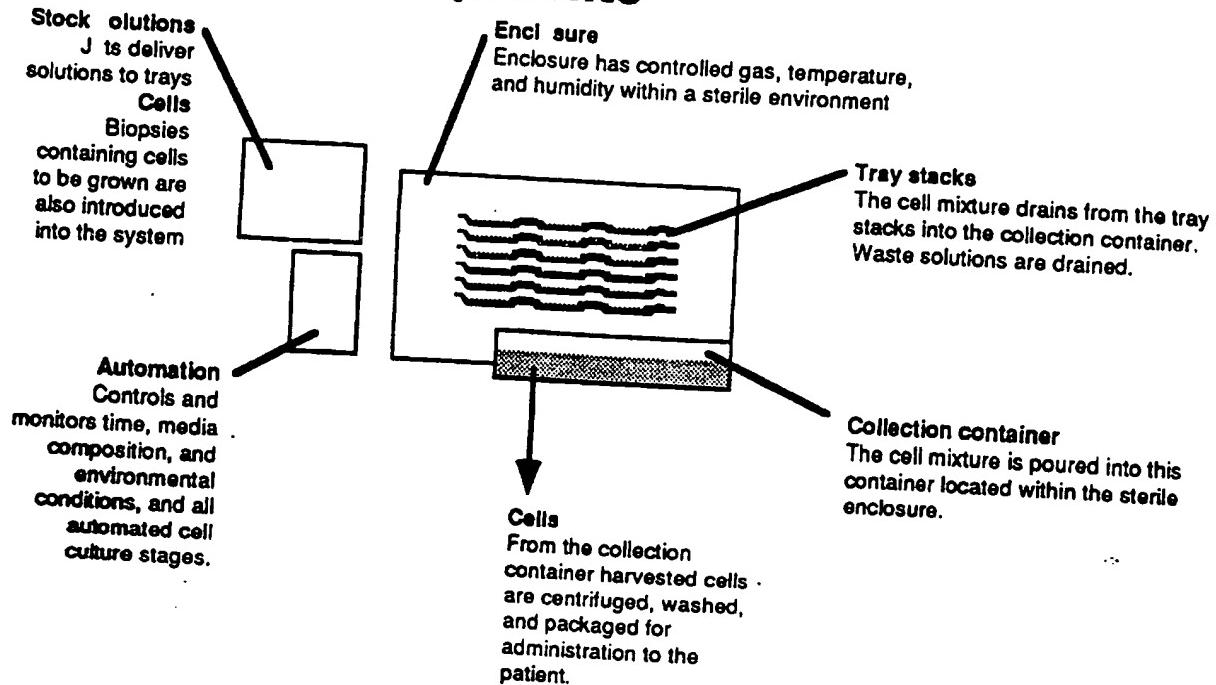
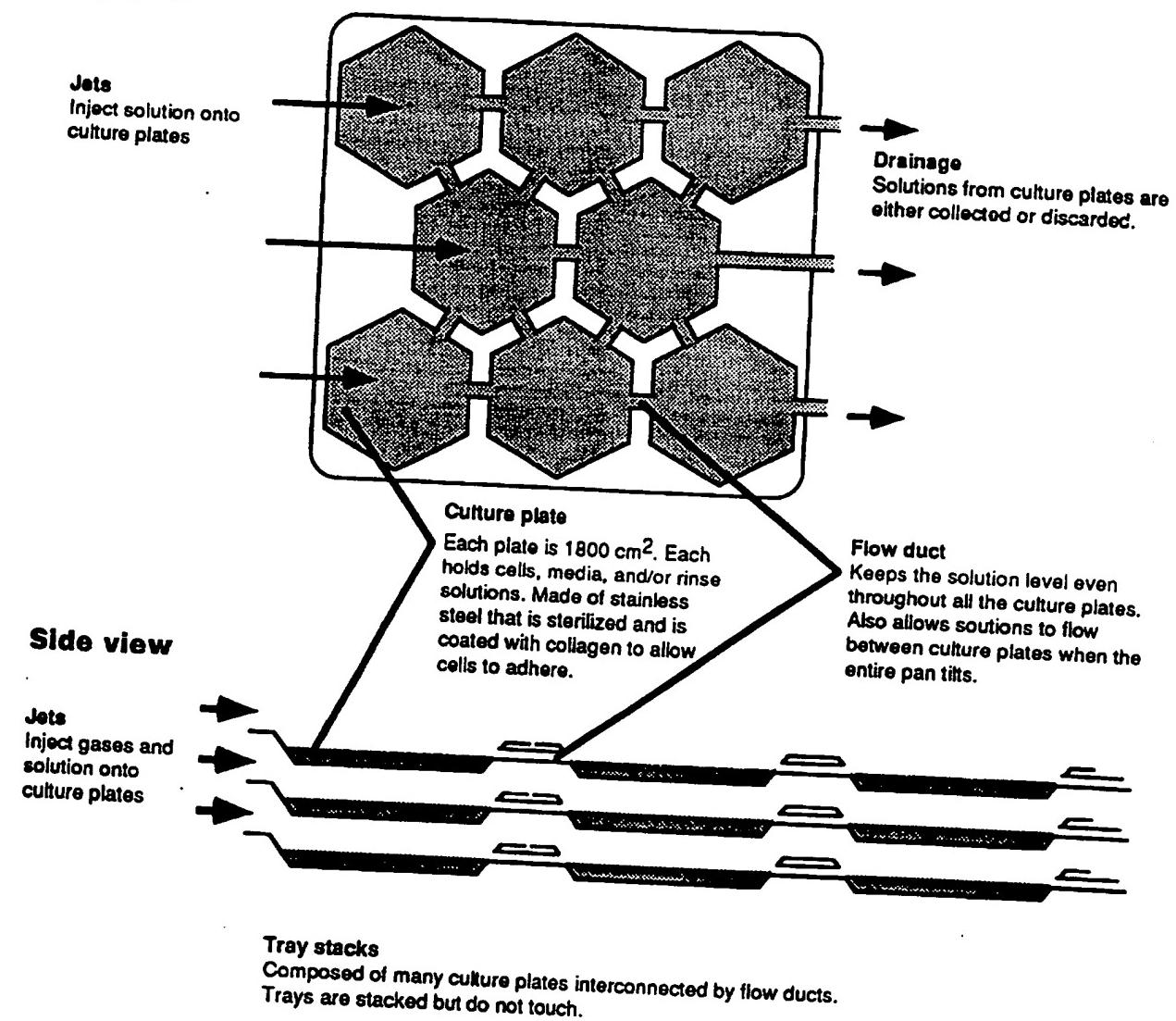


FIG.14

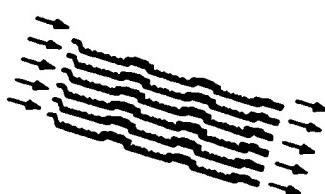
Culture trays

15 / 20

Top view

**Automated actions**

Fill
Jets fill trays with solution



Wash and drain
Tray stacks tilt to allow solutions to drain during washing or media changes. Jets add fresh solution to trays while wash solutions are drained and discarded. Detached cell mixtures are collected.



Drain and collect
Tray stacks tilt to vertical to maximize detached cell collection during harvest.

FIG.15

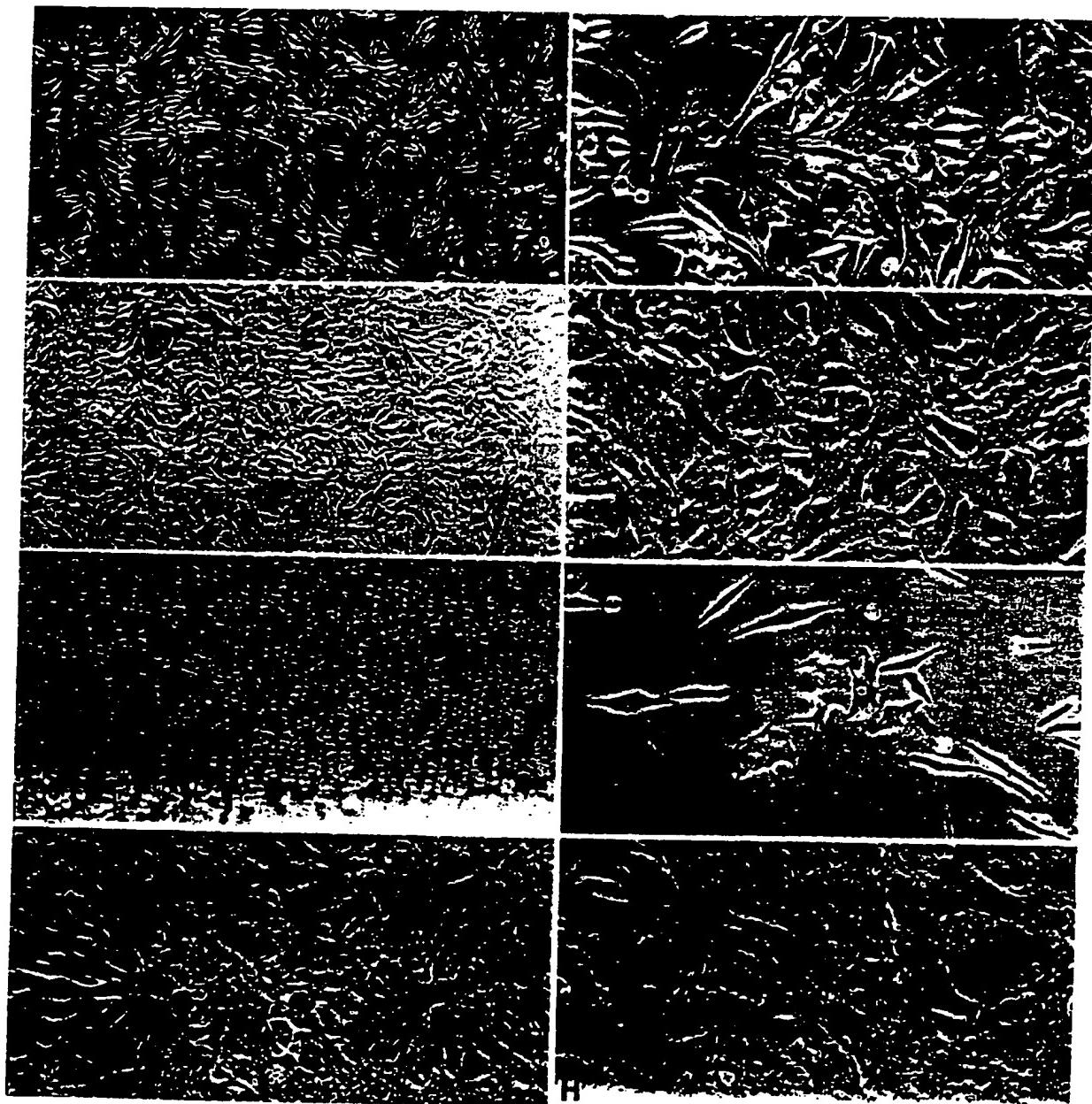


FIG.16

17/20

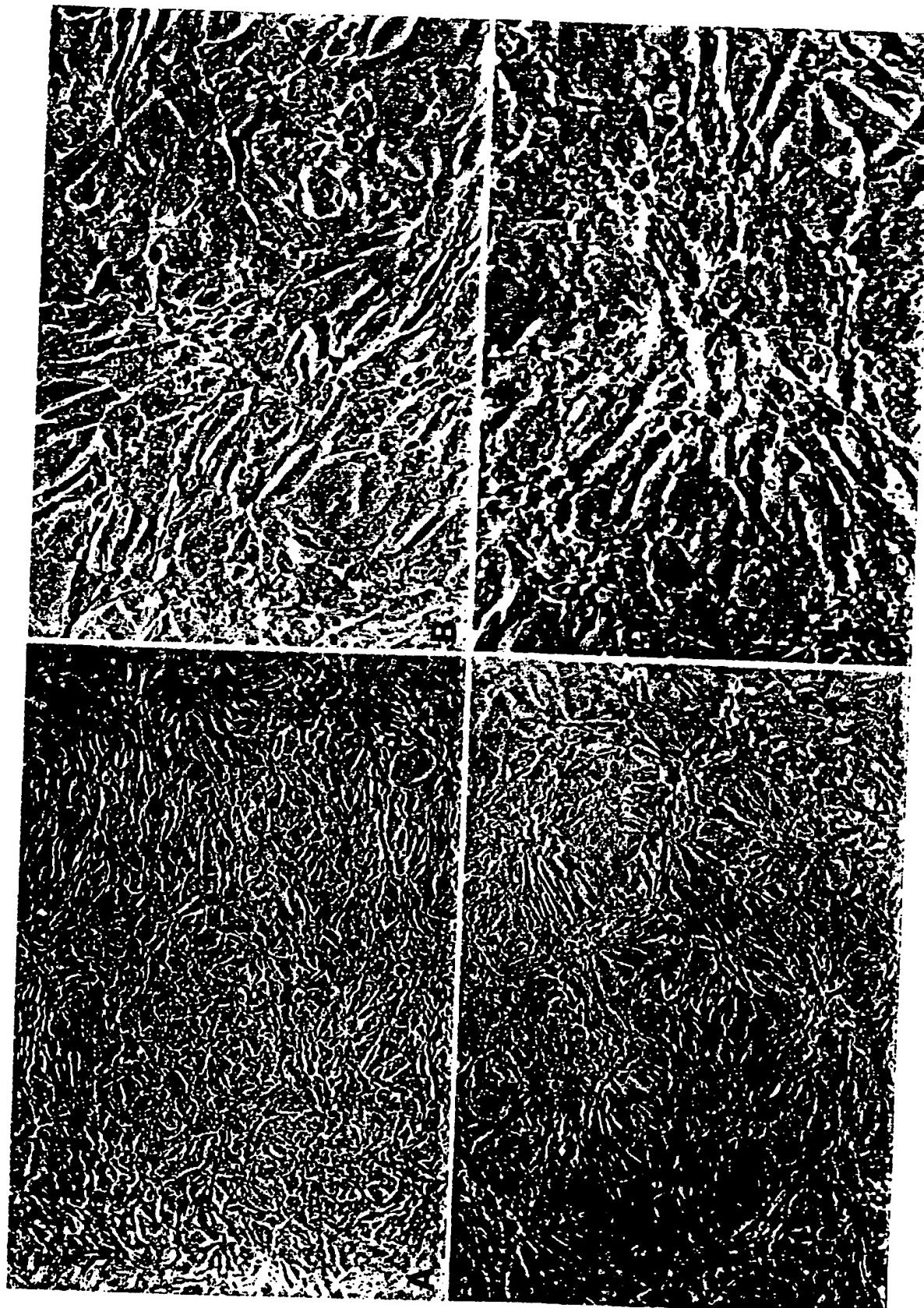


FIG. 17

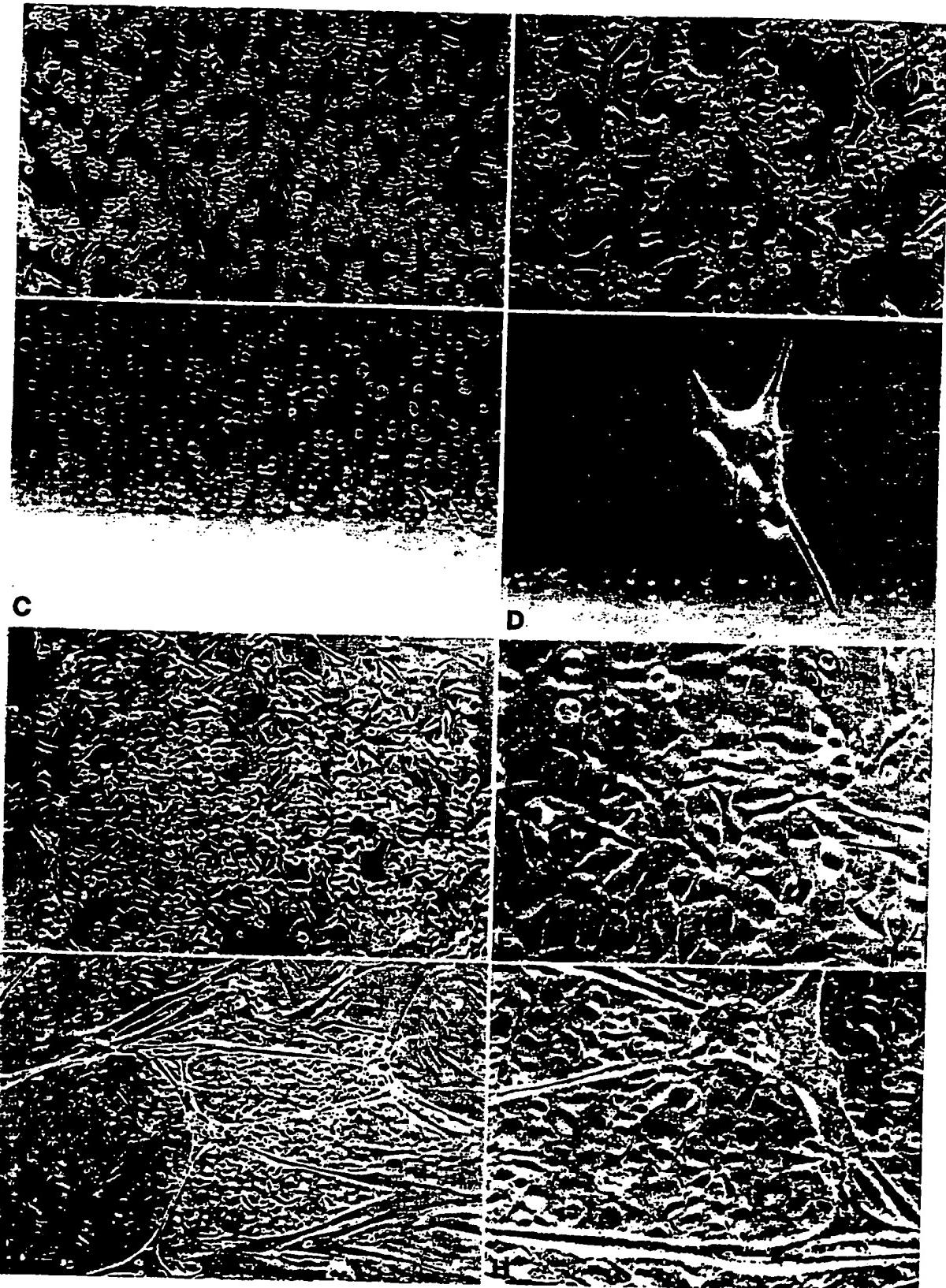


FIG.18

20/20

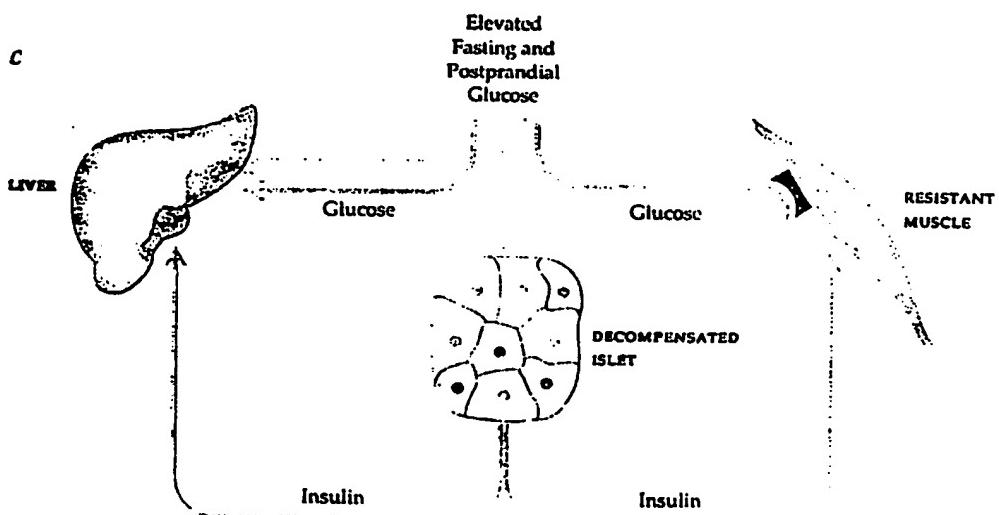
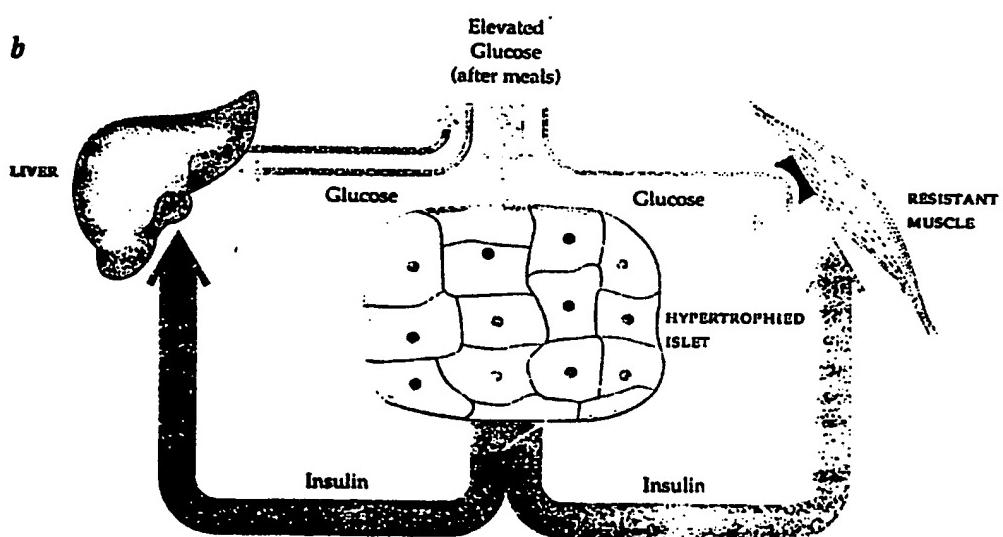
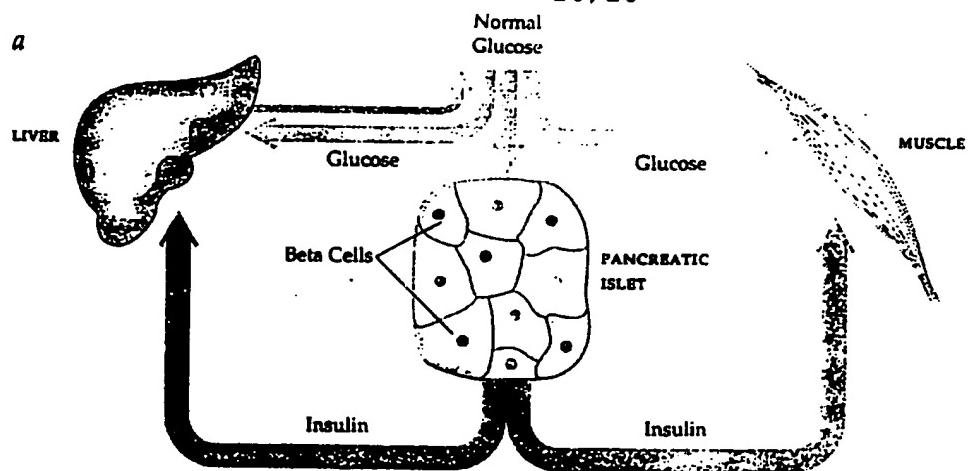


FIG. 20

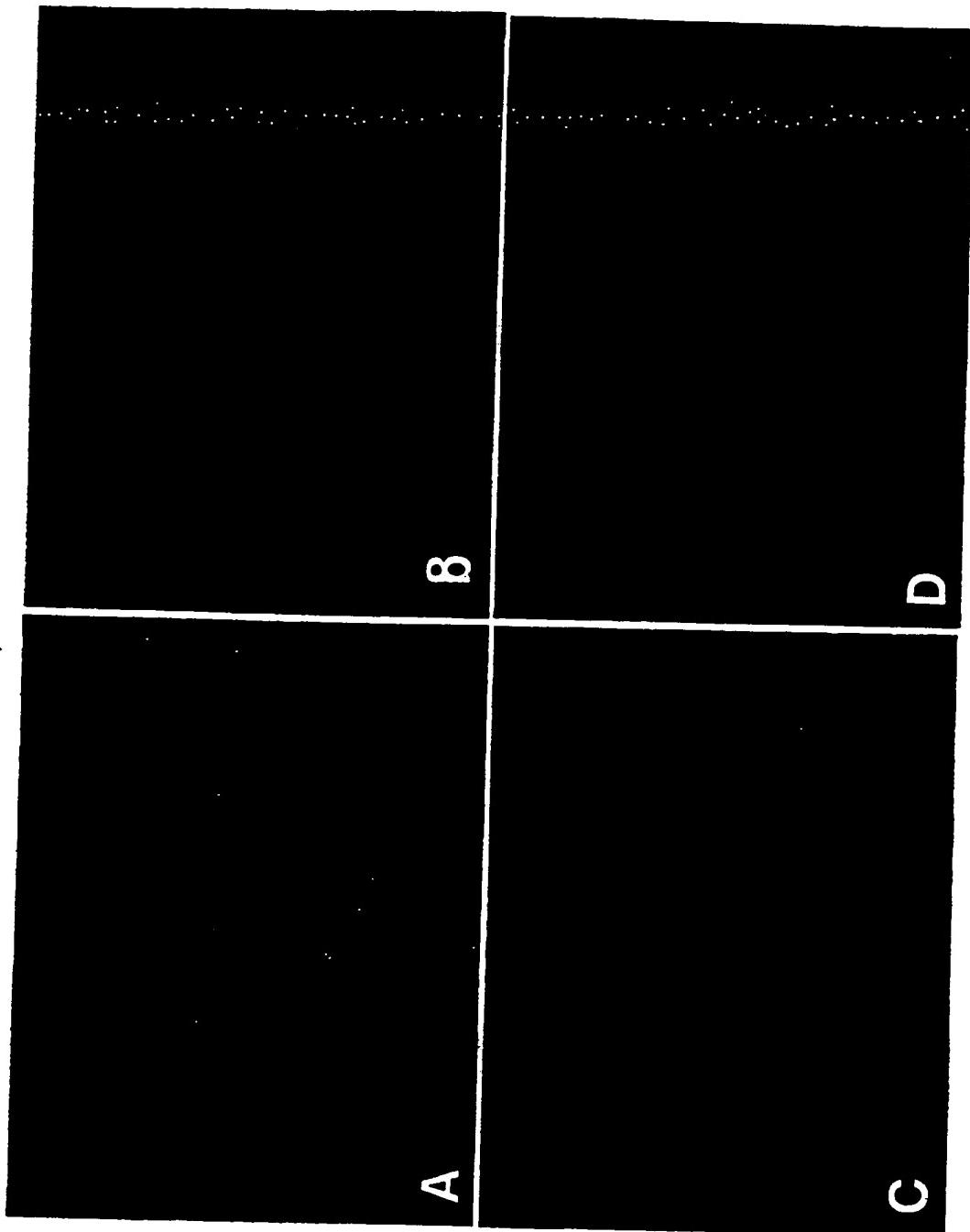


FIG. 19

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A01N 63/00; A61K 48/00, 35/34; C12N 5/00
 US CL : 424/93.2, 548; 435/240.1, 240.26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.2, 548; 435/240.1, 240.26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,130,141 (LAW ET AL) 14 July 1992, column 6, lines 10-20.	1-4 and 11-13

Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance		
E earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
I document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
02 APRIL 1996	16 APR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer <i>Deborah Crouch, Ph.D.</i>
Faxsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/16093

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-13
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/16093

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

- Group I, claim(s) 1-4 and 11-13, drawn to a method of treating mammalian diseases and cell lines.
- Group II, claim(s) 5-8, drawn to a method to control cell fusion by increasing chondroitin-6-sulfate proteoglycan.
- Group III, claim(s) 9 and 10, drawn to a method for controlling the size and shape of body parts.
- Group IV, claim(s) 14 and 15, drawn to an automated cell processor.
- Group V, claim(s) 16, drawn to a method to produce a genetically normal child.
- Group VI, claim(s) 17-19, drawn to a method for producing cardiomyocytes capable of proliferation.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: There is no apparent special technical feature that links the claims. PCT Rule 13.2 requires that for there to be unity of invention, the claims must be linked by a special technical feature. Thus, without such a special technical feature, there is no unity of invention.

This Page is inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:


 [REDACTED] BORDERS

- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT OR DRAWING
- BLURED OR ILLEGIBLE TEXT OR DRAWING
- SKEWED/SLANTED IMAGES
- COLORED OR BLACK AND WHITE PHOTOGRAPHS
- GRAY SCALE DOCUMENTS
- LINES OR MARKS ON ORIGINAL DOCUMENT
- REPERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- OTHER: _____

**IMAGES ARE BEST AVAILABLE COPY.
As rescanning documents *will not* correct images
problems checked, please do not report the
problems to the IFW Image Problem Mailbox**